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The Memorandum of Understanding between the Ministry of Health of Belize and USUHS was signed in December 1989. Two USUHS MTM&H students conducted research and obtained clinical experience in country. In August 1990, Jorge Palanco, MD received the MPH from USUHS. The renovation of the CML was completed in October 1990. The dedication of the CML and the ERC took place in November 1990. The preliminary analysis of the sera obtained in the febrile illness study indicate a significant prevalence of antibodies to many arboviruses. An outbreak of leishmaniasis in the BDF was investigated. The information obtained was incorporated in preventive measures, resulting in a significant decrease in cases. During the study of the ecology of mosquito fauna, a total of 2600 individually reared specimens (with larval and/or pupal skins) were obtained from 226 individual collections. Specimens from approximately 90 collections have been identified. Preliminary analysis of plants associated with the aquatic environments demonstrate major clusters of habitat-types

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FOREWORD

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BG In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

BG For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

BG In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Ryan C. Richt 2/13/91

PI Signature Date

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INTRODUCTION

The objective of this grant is to establish, operate and manage research and teaching programs in overseas locations where the USUHS has established, or is in the process of establishing, bilateral research agreements. These centers are to serve as sites wherein research projects of programmatic interest to the USAMRDC in the field of tropical infectious diseases can be conducted by USAMRDC and USUHS personnel in collaboration with host national counterparts. The program also provides the opportunity to transfer technology to the host-country scientists and technicians through short-term and degree-granting programs. It also provides USUHS medical students, Master of Tropical Medicine and Hygiene students and Doctoral Candidates in Medical Parasitology and Vector Biology opportunities to obtain practical experience with tropical infectious diseases of the Western Hemisphere.

In Belize, the primary objective is to establish and maintain an Epidemiological Research Center (ERC) for infectious disease research and teaching in the Ministry of Health, Central Medical Laboratory (CML), Belize City.

The research objectives are:

a) Determine the etiology of acute febrile illnesses and jaundice; determine antibody prevalence against various arthropod-borne viruses (e.g., EEE, VEE, WEE, SLE, MAY, dengue, YF, VSV, etc.), leptospirosis, hepatitis A, B, C, D, by age, sex, ethnic group, and geographical location; maintain surveillance for epidemic disease due to arthropod viruses, especially dengue.

b) Determine prevalence of HIV and HTLV-1 infections in selected populations.

c) Determine patterns of drug resistance of *Neisseria gonorrhea* in various regions.

d) Determine patterns of malaria transmission; maintain surveillance for chloroquine-resistant *Plasmodium falciparum.*

e) Determine vectorial capacity of putative malaria vectors.

f) Address epidemiologic targets of opportunity (e.g., leishmaniasis in the Belize Defense Forces (BDF); causes of febrile illnesses in British Forces Belize (BFB), U.S. Army Corps of Engineers, etc.).

g) Determine the effectiveness of repellants and fabric impregnants for protection of deployed troops from endemic vector-borne diseases.

h) Validate the remote sensing models developed for use in predicting temporal and spatial changes in malaria vector abundance in Mexico, in a second ecologically similar area.

The majority of the effort during this first year has been directed at remodeling of the CML, the establishment of the ERC and the development protocols; some research was accomplished in three areas: fevers of unknown origin, leishmaniasis, and disease surveillance in a U.S. Army Corps of Engineers which was building a bridge for the new intercoastal highway.

Background

Belize is located on the eastern coast of Central America at the base of the Yucatan Peninsula, surrounded on the west and north by Guatemala and Mexico and on the east by the Caribbean Sea. The jungle covered Maya Mountains occupy the southwestern portion of the country, rising to 1122 feet at Victoria Peak; the remainder of the country is low, crop or scrub-covered coastal plains. Belize was founded as a buccaneer settlement and entrepot. Today, Belize is an English-speaking country, having gained its independence from Great Britain in 1981. The population is estimated at approximately 175,000 people, and is made up of a mixture of Mayans, Garifuna (Afro-Amerindian), Blacks, East Indians, Creole and Caucasians. About half of the population resides in the major City, Belize City, located on the Caribbean coast. The capitol, Belmopan (population approximately 5000) is inland and was built as a Federal District after a devastating hurricane in 1961 destroyed the then capitol, Belize City. There are several other small cities; i.e., Punta Gorda, Stann Creek, Hill Bank, Orange Walk, San Ignacio and Indian Church, scattered through the coastal plain.

Medical care is provided by a socialized medical system and is centered around local health clinics and district hospitals in the smaller cities and a large central hospital in Belize City. Emergency cases (largely surgical) are brought to the Belize City Hospital for care by air ambulance, provided to the government by one of the Christian groups that has established missions throughout the country. The Belize City Hospital was built about 1930 and is a two-story building backed on the sea. It has about 200 beds, of which about 60% are dedicated to acute surgical patients. The hospital is divided into male, female and pediatric wards for both

surgery and medicine. There are, in addition, neonatal and intensive care wards. A small biochemical and bacteriology laboratory used for acute diagnostic procedures is located in the hospital. The majority of diagnostic and public health laboratory procedures are performed at the Ministry of Health, Central Medical Laboratory (CML) located about 3 miles north of the city. Current laboratory capabilities include: malaria smears (approximately 29,000 per year), bacteriologic cultures, routine biochemistries, and HIV antibody testing using commercially available ELISA kits.

Febrile Illnesses

Little is known about infectious diseases specific to Belize itself. Based upon limited information from Belize and other Central American countries, it may be inferred that in Belize, tropical infectious diseases are common. Yellow fever has been known to occur in the Yucatan[1], dengue and malaria are endemic in Belize[2], and cutaneous leishmaniasis, in almost epidemic numbers, has been reported in British troops stationed in Belize[3]. Cutaneous and visceral leishmaniasis have been reported in nearby Honduras and Guatemala[4]. Leptospirosis, and Venezuelan equine and St. Louis encephalitis have also been found in neighboring countries[5]. Enterically transmitted non-A, non-B hepatitis has recently been identified in Mexico. The Statistics Department at the hospital reported yearly admissions over the past several years for enteric fever of 4 to 6 cases; jaundice, 60 cases; and fever of unknown cause, about 150 cases. A great deal needs to be done to determine the prevalence and the incidence of tropical infectious diseases agents in Belize.

Cases of unexplained fever were selected by trained Belizean collaborators from the patients over 12 years of age presenting at the Belize City, San Ignacio and Orange Walk Hospitals. Patients with sickle cell disease, meningitis, dysentery, or evidence of peritonitis, wound infection, pneumonia, tuberculosis or HIV infection were not included in the study. Thick and thin malaria films were prepared from a finger stick and examined. Patients positive for malaria were listed but not studied. Patients selected for the study were divided into two groups, those with and those without jaundice. A systematic clinical exam was performed and blood was obtained for diagnostic tests. Sera were analyzed for the presence of antibodies to arthropod-borne viruses, leptospirosis and hepatitis.

Leishmaniasis

Cutaneous leishmaniasis is a zoonotic disease transmitted to man by human-biting female phlebotomine sand flies. Many *Leishmania* strains belonging to at least four species are capable of causing human disease. The clinical manifestations of the infection are primarily species-dependent, but a number of poorly defined host factors may influence disease expression. Flagellated promastigote-stage leishmanial parasites develop in the gut of female sand flies and are transmitted to the vertebrate host during a blood meal. Promastigotes rapidly parasitize macrophages, convert to the intracellular amastigote stage, and multiply. In the absence of specific immunity, they circulate to the regional or systemic reticuloendothelial system and ultimately cause sores and other manifestations after an incubation period of 1-6 weeks or longer.

Cutaneous leishmaniasis occurs in China, India, the Mediterranean basin, Africa, and Central/South America, but recently cases have also been reported in the southcentral United States. In the Old World, leishmaniasis is primarily caused by infection with *L. tropica*; in the New World, the main species are *L. mexicana* and *L. braziliensis*. Over a dozen different strains and subspecies have been found to be capable of causing human disease.

In the New World particularly, cutaneous leishmaniasis presents with skin lesions as a major manifestation, but regional lymphatic chains are frequently involved as well . Typically, erythematous macules appear at the inoculation sites up to months after infection, followed by papules. The papules subsequently become nodular and may ulcerate to form well circumscribed ulcers with indurated margins and necrotic eschar-covered bases. In the absence of bacterial superinfection, which is unusual, the lesions are not painful. Satellite lesions may be found a small distance from the primary ulcers in otherwise normal-appearing skin. Some strains appear to cause a milder form of the disease, and the primary lesions may not ulcerate but instead present as nodules, papules or eczematous plaques. Nontender nodules and inflamation can develop along draining lymphatics. Regional lymphadenopathy is common, and parasites can be isolated from these nodes. Metastatic spread in the New World is generally associated with *L. braziliensis* and may occur months or years after acquisition in up to 80% of infections. Infections caused by *L. mexicana, L. peruviana,* and some strains of *L. braziliensis* are thought to cause localized disease without mucous membrane involvement. Spontaneous healing appears to be quite common with *L. tropica, L. major* and *L. mexicana,* except when the ear (pinna) is involved.

Leishmania mexicana is primarily distributed in Mexico, Belize and Guatemala. It is commonly (in 40% of cases) associated with lesions limited to the pinna of the ear and classically occurs in those harvesting gum from

chicle plants, hence the name, "chiclero's ulcer." Forest rodents are the natural reservoir for *L. mexicana*. *Leishmania braziliensis* is thought to occur in Guatemala, though its presence is not as well documented as that of *L. mexicana*. In the Old World, cutaneous leishmaniasis is typically ulcerative and in many cases heals spontaneously within several months. Spontaneous healing of New World lesions is much less predictable. Certain South and Central American species, notably *L. braziliensis*, may result in a slowly healing primary ulcer and the late development of a mutilating infection of the upper respiratory tract (mucocutaneous leishmaniasis or espundia). A rare form of leishmaniasis known as diffuse cutaneous leishmaniasis is characterized by massively parasitized nonulcerated nodules, specific cutaneous anergy, and a poor response to therapy. It occurs in both the New and Old Worlds. Serious illness with leishmaniasis has been reported as a manifestation of HIV infection.

The differential diagnosis of cutaneous leishmaniasis includes pyogenic bacterial, mycobacterial (*Mycobacterium marinum*), fungal (blastomycosis, sporotricosis, histoplasmosis) and spirochetal (yaws and syphilis) infections, plus lupus erythematosis, sarcoid and malignancy.

Recent reports to the Defense Attache Office, U.S. Embassy, Belize, by local military and civilian authorities indicated that there was a substantial increase in the number of cutaneous leishmaniasis cases within the Belize Defence Force (BDF) located in the San Ignacio outpost. The Defense Attache requested the assistance of the Preventive Medicine Department, USUHS, on behalf of the Medical Corps of the BDF in assessing the extent of the epidemic and development of appropriate management strategies. The objectives were:

1. To ascertain the current status of the problem by assessing some 17 cases, reviewing baseline trends of leishmaniasis in Belize, and determining risk associations for infections.
2. To ascertain current military and civilian resources available for the diagnosis, surveillance, treatment and prevention of cutaneous leishmaniasis.
3. By making available state-of-the-art diagnostic techniques, to make certain of the diagnosis of cutaneous leishmaniasis, and to document the clinical spectrum of disease and the vector species and its distribution.
4. To estimate risk factors.
5. To recommend unit and individual preventive measures.
6. To assist the BDF and Ministry of Health in developing a medical surveillance system for case detection.

A multi-disciplined team, Team Leader (USUHS), Entomologist (WRAIR), Epidemiologist (USUHS) and Clinical Advisor (WRAIR), was deployed to Belize to assist the BDF Medical Corps and the Ministry of Health in responding to this epidemic and to prepare them for future epidemics.

The BDF patient data were collected at Price Barracks, Ladyville. The BFB data were obtained at the Airport Camp Hospital. All military cases and two civilian cases occurred in Cayo District. The meterologic data were provided by the weather station at the Belize City Airport. Epidemiologic and entomologic data were collected throughout the Cayo District. A total of 15 of the 18 reported patients were seen. Clinical and epidemiological data were obtained from all cases. There was photographic documentation of all lesions.

Fifty-six needle-aspirate cultures were obtained from the 15 BDF and 2 civilian cases. The aspirates were inoculated into NNN and Schneider's media and stained by indirect fluorescent antibody.

U.S. Army Corps of Engineers

The 20th Engineer Company of the US Army Corps of Engineers from Fort Campbell, Kentucky, deployed a unit to Belize in May 1990 to build a bridge over the Mullins River as part of the costal highway. Blood samples were drawn prior to departure from the US and the unit was briefed on the endemic diseases and their prevention. Blood samples were collected from patients reporting with signs of illness and from all soldiers before their returning to the US in June 1990. Samples are to be analyzed for the presence of antibodies to diseases of interest at USAMRIID.

Ecology of Mosquito Fauna

Belize, at 15-19° N latitude, is located in the subtropical geographical belt of Central America. The warm ambient temperatures favor malaria transmission throughout the year. The mean monthly minimum temperatures vary from 16-17° (C) in the winter to 24-25° (C) in the summer. Annual average rainfall is 1347 mm for the northern Corozal District, 1323 mm for the more central Cayo District, and 4526 mm for the southern Toledo District. The dry season occurs between January and April to May, with the shortest dry season occurring in the southcentral region.

Relief varies from areas below sea level to a mountain peak of 3806 feet. The coastal area is generally flat and marshy, with extensive areas of mangrove swamp and other types of marsh vegetation. The most pronounced feature of the mainland is the "Maya Mountains" in the southcentral part of the country. These mountains are recognized as the oldest land surface in Central

America and the Mountain Pine Ridge plateau is thought to be a remnant of the ancient land surface.

The primary objective of the faunistic survey was to collect taxonomic series of mosquito species found in Belize, with secondary emphasis on quantifying the abundance of anophelines within various aquatic habitats. Habitats that produced few mosquitoes were sampled as aggressively as were the more productive habitats. Dr. Rejmankova collected plants, recorded observations on plants and plant abundance for each habitat sampled, and collected water samples for physical/chemical analyses. The latter efforts were targeted to provide background information about the habitat preferences of *An. albimanus* that might be of particular interest to the Belize malaria control program personnel.

BODY

Administrative

The Memorandum of Understanding between the Ministry of Health (MOH) of the Government of Belize and the Uniformed Services University of the Health Sciences (USUHS) was signed in December 1989 by the Honorable Robert G. Rich, Jr., Ambassador of the United States of America and Theodore Aranda, Ph. D., Minister of Health and Urban Development. The cost reimbursable contract agreement between Henry M. Jackson Foundation for the Advancement of Military Medicine (HJF) and the Ministry of Health of the Government of Belize, Central America, was also signed in December 1989.

Two USUHS MTM&H students have participated in research efforts and had the clinical experience of working in a third world country. In August 1990, Jorge Palanco, MD received the MPH from USUHS, returned to Belize and is the Program Manager for AIDS and Malaria and Vector-Borne Disease Control.

The renovation of the CML was initiated in February 1990 and completed in October 1990. The dedication of the CML and the ERC took place in November 1990.

Protocols have been prepared and submitted for consideration by the USAMRDC Oversight Committee covering many of the research objectives. The planned implementation timeline is shown in the Conclusions.

Febrile Illnesses

Demographic data and sera were collected on 63 patients and, to date, sera from 40 patients have been analyzed. Results of this analysis are shown in Table 1.

Table 1. Prevalence of viral antibodies in patients in FUO protocol.

	LOCATION		
ANTIBODY	ORANGE WALK	BELIZE CITY	TOTAL
EEE	2	3	5
VEE	7	8	15
WEE	2	0	2
MAY	5	4	9
DEN 2	11	13	24
DEN 4	13	14	27
SLE	14	11	25
YF	4	2	6
VSVI	1	0	1
VSVNJ	3	4	7
TOTAL REACTORS	62	59	121
TOTAL PATIENTS	39	33	72

Leishmaniasis

The characteristics of the leishmaniasis outbreak are summarized in Tables 2-4. The cases were distributed amongst 5 different companies. Three of the companies were assigned to Camp Bellazario, base for jungle/border patrols, for a 3-month tour during the months of February-August 1989. The other 2 companies had only been on jungle/border patrol during annual summer camp during the first two weeks of July at a site within Cayo District. Insect repellant (DEET) was normally available; however, the supplies were exhausted in June and were unavailable during summer camp. All cases were men with a mean age of 22.4 years. There were multiple lesions on arms/hands, legs/feet, head/neck and torso in 5. The mean size of the primary lesions was 24x24 mm, with the majority equally distributed on arms, legs and torso. Patients had been treated with one or more of the following: Glucantime, 15% paromomycin cream and traditional medicine (herbs, leaf and root extracts, topical acid and/or burns). All BDF *Leishmania* cultures were negative. An aspirate from a civilian seen in San Ignacio who had not been treated grew *Leishmania mexicana mexicana*. Two patients were positive by DFA with monoclonal antibodies to *Leishmania*. Five species of sand flies were identified; 3 were man-biters, with *Lutzomyia shannoni* predominating.

The epidemic curve is shown in Figure 1. Three factors were apparently responsible for the outbreak: (1) the months of May and June were unusually dry, which enhanced the survival of adult sandflies, (2) the summer camp in July was in an area endemic for leishmaniasis and (3) failure to use topical repellant.

Prior to this year's (1990) summer camp, the troops were thoroughly briefed on preventive measures and there was plenty of insect repellant. Five months following camp at the same location, there have been only 2 cases in the BDF. The BFB, on the other hand, have had 16 cases in the same period.

U.S. Army Corps of Engineers

The majority of patients seen complained of insect bites and sunburn. There were no reported cases of febrile illness. Serological examinations have not been accomplished yet.

Table 2. Characteristics of the Outbreak. Cutaneous Leishmaniasis in the Belizean Defence Force.

		# (%)
Total number of Belizean Defence Force troops (Infantry)		600
Total Number of Cases of Cutaneous Leishmaniasis; Oct. 1989.		18 (3)
Cases reviewed by EPICON.		
	BDF	15
	CIVILIAN	2
Mean age of Cases.		22.4
Rank Distribution:	Rank	No. (%)
	Officer	1 (5.5)
	Sgt.	2 (11)
	Cpl.	2 (11)
	Lcpl.	2 (11)
	Pvt.	11 (61)
Company Distribution:	Company	No. (%)
	Alpha	8 (9)
	Echo	2 (2)
	Fox	4 (4)
	Support	5 (5)
	Headquarters	1 (1)

Company Rotation Thru Camp Bellazario (Cayo Region).

(Bellazario is base for Border/Jungle Patrols.)

Company	Months
Echo	15 Feb.- 15 May
Alpha	15 May -15 Aug.
Fox	15 Aug - present
Support	Annual Camp *
Headquarters	Annual Camp *

* Annual Camp occured during the first two weeks in July. This is the only Battalion Strength operation every year. Mountain Pine Ridge , located in the Cayo District, was the site selected for Annual Camp 1989.

Table 3. Clinical Characteristics. Outbreak of Cutaneous Leishmaniasis in the Belizean Defence Force (1989).

Distribution of Lesions by Company:

Company	Single Lesions (%)	Multiple Lesions(%)	Total(%)
Alpha	2 (20)	3 (43)	5 (30)
Fox	3 (30)	0 (0)	3 (18)
Echo	1 (10)	1 (14)	2 (12)
Support	2 (20)	2 (29)	4 (24)
HQ	0 (0)	1 (14)	1 (6)
Civilian	2 (20)	0 (0)	2 (12)
Total	10 (100)	7 (100)	17 (100)

Anatomical Distribution of Lesions:

Site	# Single(%)	# Multiple (%)	Total (%)
Arms/Hands	5 (50)	4 (21)	9 (31)
Legs/Feet	3 (30)	6 (32)	9 (31)
Head/Neck	2 (20)	3 (16)	5 (17)
Torso	0 (0)	6 (32)	6 (21)
Total	10 (100)	19 (100)	29 (100)

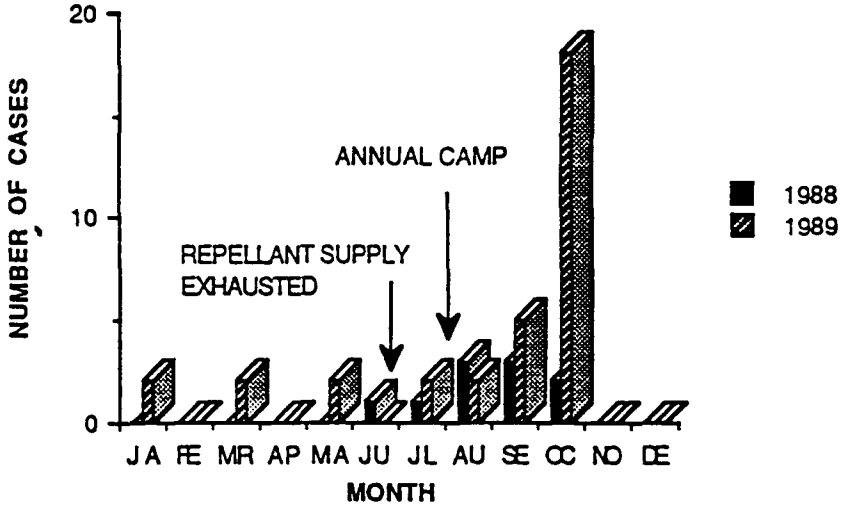
Table 4. Clinical Characteristics. Outbreak Investigation of Cutaneous Leishmaniasis in the Belizean Defence Force.

Antatomical Distribution of Lesions (primary lesions only):

SITE	NUMBER	(%)
Arms/Hands	7	(33)
Legs/Feet	5	(24)
Head/Neck	3	(14)
Torso	6	(29)
Total	21	(100)

Mean Size of Primary Lesions (in mm). 24x24

Figure 1. Cases of Cutaneous Leishmaniasis in the BDF



Ecology and of Mosquito Fauna

A total of 2600 individually reared specimens (with larval and/or pupal skins) were obtained from 226 individual collections. Specimens from approximately 90 collections have been identified.

Comparable surveys have previously been performed by Drs. Harry Savage and Rejmankova in Tapachula, Mexico, and data from their surveys are available for comparisons of habitat availabilities and habitat/mosquito associations.

Collection sites were widely distributed in the northern half of Belize (Figure 2). Preliminary analysis of plants associated with the aquatic environments demonstrate major clusters of habitat-types (Rejmankova, *et al.* 1991 [Figure 3]). A comparison of dominant plant species found in surveys conducted in Belize and Mexico is presented in Table 5.

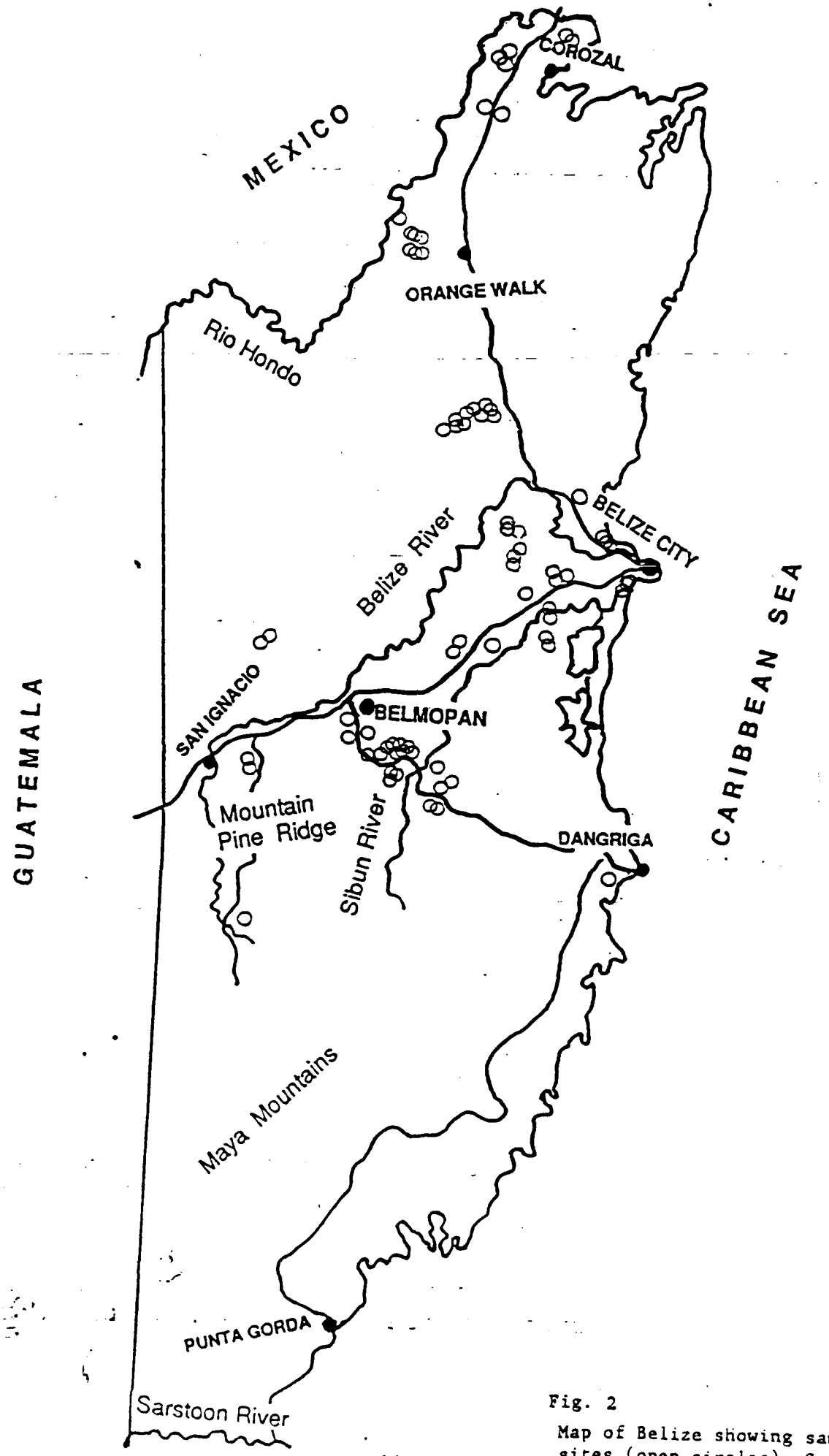


Fig. 2
Map of Belize showing sampling sites (open circles). September 1990

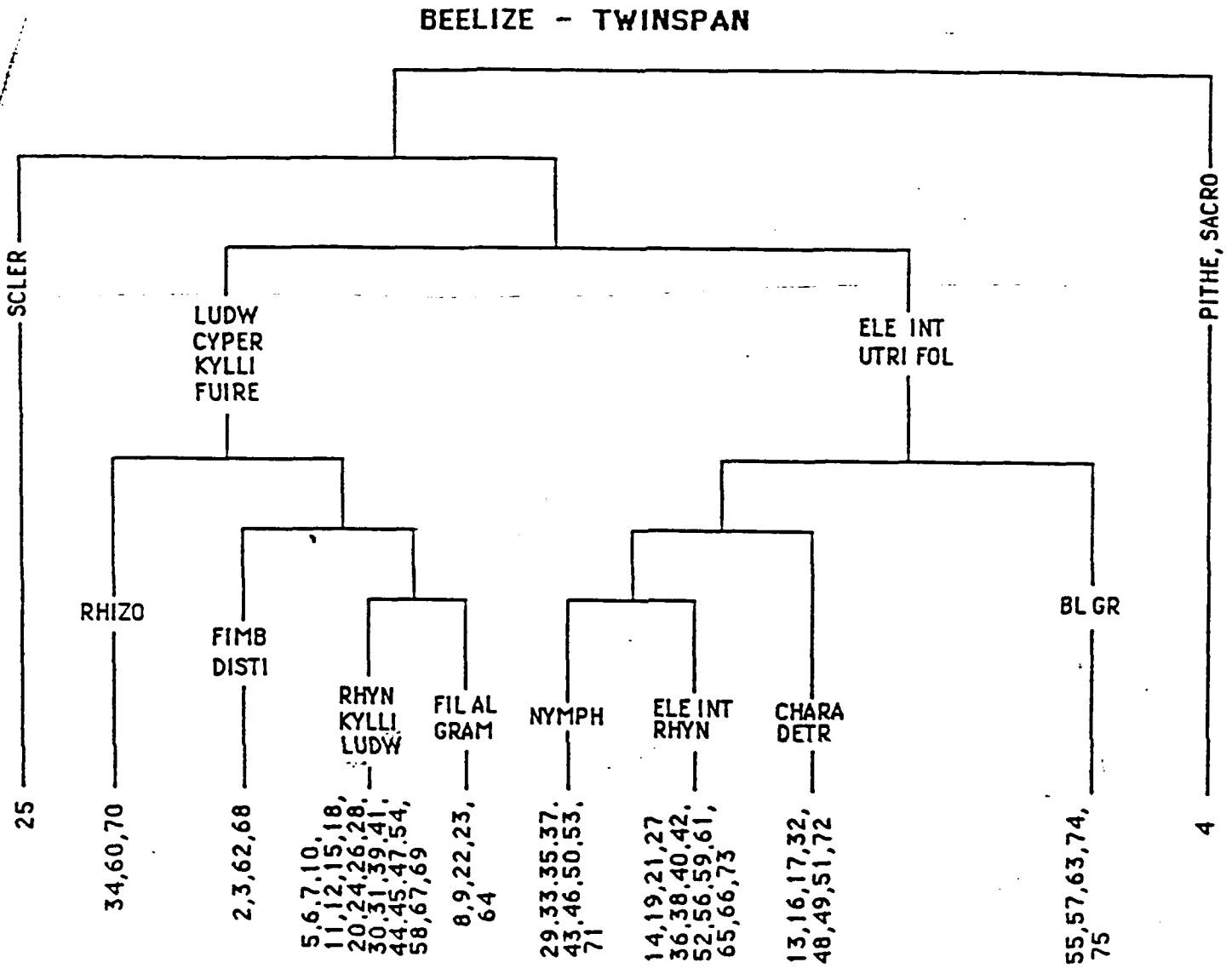


Fig. 3 TWINSPAN classification of mosquito larval sampling sites based on plant species abundances. Numbers indicate sampling sites.

TWINSPAN - two-way indicator species analysis (Hill 1979) is the divisive hierarchical classification. The set of samples is successively divided and each division has indicator species characteristic for each dichotomy. Belize data set: 72 plant species, 75 samples

Hill, M. O. 1979. TWINSPAN - A FORTRAN program for multi-variate data in an ordered two-way table by classification of the individuals and attributes. Ithaca, N.Y., Cornell University.

Table 5. Comparison of dominant plant species from Tapachula and Belize sampling sites

CHIAPAS	BELIZE
Emergent	
Gramineae	
Hymenachne amplexicaulis	Hymenachne amplexicaulis
Paspalum sp.	Paspalum sp.
-	Paspalum virgatum
Panicum sp.	-
Pennisetum sp.	-
Echinochloa sp.	-
Cynodon dactylon	-
Jouvea straminea	-
Gramineae sp.	Gramineae sp.
-	Distichlis spicata
-	Leptochloa sp.
Cyperaceae	
Fimbristylis spadicea	Fimbristylis spadicea
Cyperus sp.	Cyperus rotundus
-	Cyperus odoratus
-	Kyllinga sp.
-	Fuirena umbellata
-	Rhynchospora cyperacea
-	Rhynchospora robusta
-	Eleocharis intersticta
-	Eleocharis caribea
-	Eleocharis cellulosa
Typha domingensis	Typha domingensis
Broadleaf	
Ludwigia octovalvis	Ludwigia octovalvis
Pontederia sagittata	-
Batis maritima	-
Sagittaria lancifolia	-
Thalia geniculata	-
Colocasia esculenta	-
Crinum erubescens	-
Mimosa sp.	-
-	Bacopa monnieri
-	Spilanthes sp.
-	Justicia sp.
Rhizophora mangle	Rhizophora mangle
Floating	
Eichhornia crassipes	-
Heteranthera sp.	-

Floating (cont.)	
Pistia stratiotes	-
Salvinia minima	-
Lemna sp.	Lemna sp.
Nymphaea ampla	Nymphaea ampla
-	Limnanthemum humbolti
Algae	
Filamentous	*Filamentous*
-	Blue green mats
Submerged	
-	Utricularia foliosa
-	Utricularia resupinata
-	Utricularia purpurea
-	Chara sp.
-	Naias guadalupensis
-	Mayaca fluitans

CONCLUSIONS

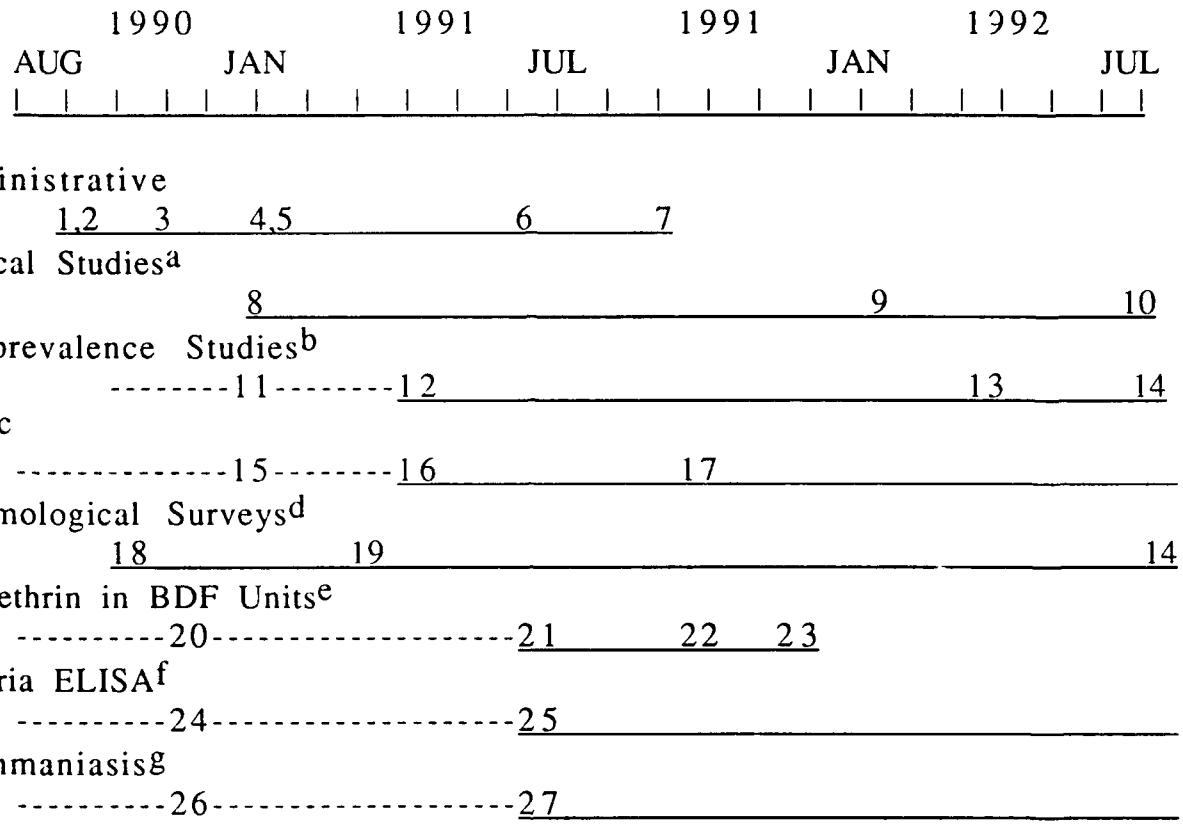
Too few patients have been studied to date to draw inferences about major causes of FUO and jaundice; however, the prevalence of antibodies to a wide range of infectious disease agents of interest is high. Seroprevalence surveys are necessary to determine the geographic regions and populations where additional surveillance and prevention efforts are warranted. A revitalization of this effort will be undertaken in January 1991.

Leishmaniasis is endemic throughout the forested areas of Belize. An abstract of the report of the leishmaniasis outbreak in the BDF, which was presented at the Annual Meeting of the American Society of Tropical Medicine and Hygiene, New Orleans, LA, 5-9 November 1990, is shown in Appendix 1. The incidence of leishmaniasis in BDF and BFB troops should continue to be monitored, and when necessary, interventions should be introduced. A comprehensive study of leishmaniasis should be started to define the epidemiology of the disease more completely in Belize.

A detailed analysis of mosquito larval associations with habitats (defined by individual plant species) and habitat-types (defined by common groupings of plant species) will be performed after mosquito identifications are completed. The USUHS, WRBU and UC-Davis team of investigators hope to return to Belize in March or April for a dry season mosquito collection. These surveys will serve as the foundation for future studies of malaria and arboviral epidemiology.

The timeline for the introduction of planned studies to the ERC is shown in Figure 4.

Figure 4. Timeline for research program in tropical infectious diseases.



1. Dr. Polanco completes MPH, returns to Belize
2. Laboratory renovation completed
3. Belizean Administrator, Secretary, and Laboratory Technicians hired
4. Training of Belizeans on new laboratory equipment completed
5. U.S. Co-Director assigned
6. Establish teleconferencing capability
7. Second Belizean epidemiologist completes training
8. Rejuvenate clinical studies of FUO, jaundice
9. Completed clinical data collection
10. Complete analysis of laboratory specimens
11. Protocol written
12. Protocol approved; studies initiated
13. Study completed
14. Serologic examinations completed, data analyzed; design surveillance program, field studies

15. Protocol written
16. Protocol approved; begin establishment of STD capabilities in districts
17. Complete planning, logistics; initiate studies
18. Faunistic survey accomplished
19. Data analysis completed
20. Protocol approved
21. Planning, logistics completed; initiate study
22. Study completed
23. Data analyzed, report prepared
24. Protocol written, approved
25. Planning, logistics completed; initiate study
26. Protocol written, approved
27. Planning, logistics completed; initiate study

REFERENCES

1. Bustamante, M.E. Un descubrimiento cietifico truncado en 1912, et de la fiebre amarilla de la serva en Yucatan. 1986, *Gac. Med. Mex.* 122:263-272.

2. Freeman, K. American cutaneous leishmaniasis. 1983, *J. R. Army Med. Corps.* 129:167-173.

3. Navin, T.R., M. Sierra, R. Custodio, F. Steurer, C.H. Porter, and T.K. Ruebush. Epidemiologic study of visceral leishmaniasis in Honduras, 1975-1983. 1985, *J. Trop. Med. & Hyg.* 34:1069-1075.

4. Sanchez, J.L., E.T. Takafugi, W.M. Lednar, J.M. LeDuc, F.F. Macasaet, J.A. Mangiafico, R.R. Rosato, D.P. Driggers, and J.C. Haecker. 1984 Venezuelan equine encephalomyelitis: report of an outbreak associated with jungle exposure. 1984, *Mil. Med.* 149:618-621.

5. Tucker, R.V. What makes primary health care work in Toledo District, Belize. *1988 Abstracts NCIH Conference,*

APPENDIX

Attachment 1

Craig-P. **Krieg-R-E. Brady-W-E. Perkins-P-V. Berman-J-D. Duncan-J-F. Legters-L-J. AN OUTBREAK OF CUTANEOUS LEISHMANIASIS IN THE BELIZEAN DEFENCE FORCES, BELIZE, C.A. Belizean Defence Force, Belize, C.A., **USUHS/PMB, 4301 Jones Bridge Road, Bethesda, MD 20814-4799, WRAIR, Washington, D.C.
During October 1989, 18 cases of cutaneous leishmaniasis were reported in the Belizean Defence Force (BDF) stationed at Price Barracks, Ladyville, and Camp Bellazario, San Ignacio, Belize. An Epicon team from the United States was deployed to assist the BDF and the Ministry of Health (MOH) in the investigation. Histories were obtained on 15 of the 18 reported cases and following clinical evaluation, needle aspirates of lesions and a blood specimen were taken for cultures and serologic studies. Sand flies were collected in various locations throughout the Cayo District. The cases were distributed amongst 5 different companies. Three of the companies had been assigned to Camp Bellazario, base for jungle/border patrols, for a 3 month tour during the months of February - August 1989. The other 2 companies had only been on patrol during annual summer camp during the first two weeks of July at a site within Cayo District where border/jungle patrols were conducted out of Camp Bellazario. Insect repellant (DEET) was normally available; however, the supplies were exhausted in June and were unavailable through summer camp. All cases were men with a mean age of 22.4 years. There were multiple lesions on arms/hands, legs/feet, head/neck and torso in 5. The mean size of the primary lesions was 24x24 mm with the majority equally distributed on arms, legs, and torso. Patients had been treated with one or more of the following: glucantime, 15% paromomycin cream, and traditional medicine (herbs, leaf and root extracts, topical acid or burns). All BDF patient *Leishmania* cultures were negative. An aspirate from a civilian seen in San Ignacio who had not been treated grew *Leishmania mexicana mexicana*. Two patients were positive by DFA with monoclonal antibodies to *Leishmania*. Five species of sandfly were identified, 3 were man-biters with *Lutzomyia shannoni* predominating. Three factors were responsible for the outbreak: (1) the months of May and June were unusually dry and enhanced the survival of adult sandflies, (2) the Summer camp in July was in an area endemic for leishmaniasis and (3) failure to use topical repellant.

APPENDIX II

PULSE LABORATORY

Annual Report For Period Ending December 31, 1990

TITLE: PREVALENCE OF SAND FLY FEVER, WEST NILE, CRIMEAN-CONGO HEMORRHAGIC FEVER AND LEPTOSPIROSIS ANTIBODIES IN PAKISTANI MEN

INVESTIGATORS:

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ABSTRACT

To determine the prevalence of antibody to viral diseases known or suspected to be present in Pakistan, we studied 570 sera from three groups of adults; two of the groups were involved in outbreaks of enterically transmitted non-A, non-B hepatitis, and the third included men admitted to a hospital for evaluation of a febrile illness. IgG anti-leptospiral antibody was found in 1-6% of the subjects, with the highest rate in enlisted military personnel hospitalized for febrile illness. Only one man in the group with febrile illness had significantly elevated IgM anti-leptospiral antibody titers. However, in a group of recruits experiencing suspected non-A, non-B hepatitis, 19 (11%) of 173 had a four fold rise in IgM antibody to leptospirosis. Antibody to sand fly fever viruses was found in 43-76%. The lowest prevalence, 26%, was observed in a subset of subjects ≤19 years old attending a service academy. Antibody to West Nile virus, which causes a febrile illness with rash and encephalitis, was present in 37-46% of subjects. Antibody reactive with Japanese B encephalitis virus was present in 32-35%, but in almost all cases, it appeared to be cross-reactive with West Nile virus. All 212 specimens tested for Crimean-Congo hemorrhagic fever virus were negative. This study indicates that diseases known to be prevalent in other areas of Southwest Asia and the Middle East are also prevalent in Northern Pakistan and may impact on travelers to the area.

STATEMENT OF THE PROBLEM

The prevention, diagnosis and treatment of diseases in the Asian subcontinent requires knowledge of prevalent diseases. Several infectious agents are known or suspected to be present in this area of the world. Sand fly fever virus is transmitted by *Phlebotomus papatasi* and commonly causes a 2-4 day febrile illness \1/. West Nile virus, a flavivirus transmitted by *Culex spp.*, also causes a febrile illness, often with rash, and occasionally with encephalitis \2/. A related flavivirus, Japanese B encephalitis virus, is present in Southeast Asia and recently has caused severe outbreaks in neighboring India \3/, but it has not been detected in Pakistan. Crimean-Congo hemorrhagic fever virus, transmitted by *Hyaloma spp* ticks in nature and man to man nosocomially, also occurs in Pakistan \4/. Leptospirosis, a febrile illness transmitted through contact with infected urine, is also present in Pakistan \5/ and India \6/. We performed a serosurvey in 3 groups of patients in Pakistan to determine the seroprevalence of antibodies against these viruses and leptospires and compared our findings with data in the literature about these diseases in Pakistan and neighboring India, Iran, Iraq and Afghanistan.

METHODS

Patients: All patients in the study were men. One group consisted of 212 male military recruits undergoing training near Mardan, Pakistan, including some who developed jaundice without serologic evidence of acute hepatitis A or B \7/. Paired sera were collected on these men in October and November 1984. Another group of 192 men, 16-46 years of age, was involved in an epidemic of enterically transmitted non-A, non-B hepatitis (hepatitis E) at a service academy in Sargodha, Pakistan, in 1987 \8/. The

third group was comprised of 254 male patients age 12-75 (mean 28 years) admitted to the Military Hospital in Rawalpindi for evaluation and treatment of acute febrile illnesses between October 1986 and January 1988.

Laboratory: Viral serodiagnostic tests were performed by ELISA \9/. Viral antigens, with one exception, were grown in tissue culture cells in roller bottles. The antigen was the clarified supernatant maintenance medium collected from infected cultures after cytopathic effects reached 75-100%. The one exception was that of Crimean-Congo virus (Strain IbAr10200), which was harvested from the brains of infected mice as a 10% clarified suspension of brain material in borate saline. Prior to use, the virus was inactivated by beta propriolactone \10/. Viral antigen dilution for each assay was determined by checkerboard titrations. A whole cell detergent extract (prepared by R. R. Graham, USAMRU-ROK, APO San Francisco 96301-0424), which is thought to be broadly cross-reactive with human antibodies to all known pathogenic serovars of leptospira, was used as leptospiral antigen.

For IgG assays, viral antigens were captured onto the solid phase of PVC microtiter plates by hyperimmune mouse ascitic fluid, which acted as specific immunocapture antibody for each viral agent. Dilutions used for each hyperimmune ascitic fluid and its corresponding viral antigen were determined in checkerboard titrations. Leptospiral antigen was coated directly onto PVC plates overnight at 4^{o} C. Sera were tested in duplicate in wells coated as above and against duplicate rows of a mock antigen. Horseradish peroxidase conjugated anti-human IgG (gamma chain specific, prepared in mice, Accurate Chemical and Scientific Co., Westbury, NY) was added, and bound horseradish peroxidase was measured using ABTS (KPL, Gaithersburg, MD), read spectrophotometrically at 410 nm. The adjusted optical density (OD) of the test was

obtained by subtracting the average OD of the mock antigen-coated wells from the specific antigen-coated wells. If this value exceeded a cut-off value obtained by summing the mean and 3 standard deviations of a panel of 5 normal sera, it was considered to be positive. Positive sera were titrated by serial two fold dilutions beginning at 1:100; the last dilution that exceeded the cut-off value was considered the extinction titer.

IgM antibody was detected using an IgM capture format. Sheep anti-human Mu chain antibody IgM was coated onto the PVC plates, and IgM was captured from a 1:100 dilution of sera. Viral- or leptospiral- specific IgM was measured by reacting viral or leptospiral antigen and mock control antigens with the captured IgM; those sera containing specific IgM captured the viral or leptospiral antigen, and were then measured with an antigen detection system comprised of mouse anti-viral hyperimmune ascitic fluid or rabbit anti-leptospiral antibody, followed by the appropriate horseradish peroxidase conjugated anti-mouse antibody or anti-rabbit antibody (KPL, Gaithersburg, MD). Optimal dilutions of viral or leptospiral antigens, virus- or leptospiral-specific antibodies and conjugates were determined by sequential checkerboard titrations. Bound enzyme was quantitated at 410 nm and positive-negative determinations were calculated similarly to the IgG assay: a cut-off value was obtained by summing the mean and 3 standard deviations of the adjusted OD values of a panel of negative sera. Titers were determined by serial two fold dilution of sera from 1:100, and the last dilution which exceeded the calculated cut-off was considered the extinction titer.

RESULTS

The prevalence of IgG antibodies to leptospirosis was low. Only 3 (1%) of subjects from the service academy had IgG antibody to leptospirosis. The highest prevalence, 6%,

was seen in enlisted men hospitalized for evaluation of febrile illness. Acute leptospirosis was not clinically suspected in any of the hospitalized men, and in only one subject was a titer of IgM antibody ≥1:200 detected. Likewise, in subjects from the academy, only 3 subjects had titers of IgG of 1:100 and 1 had titers of IgM at 1:800. In contrast, in a group of military recruits that had experienced an outbreak of apparent non-A, non-B hepatitis, 34 (16%) had IgM titers ≥1:200 on a single specimen. A four fold rise in antibody titer over one month was observed in 19 (11%) of 173 with paired sera. Jaundice, a finding in leptospirosis, was reported in 9 of 212 of these recruits but only 1 of the 9 had elevated IgM anti-leptospiral antibodies in the acute serum and no convalescent serum was available.

Antibody to sand fly fever virus of both Naples and Sicilian types was prevalent in all three groups (Figure 1). Recruits at Mardan and patients with febrile illnesses had the highest prevalence of IgG antibody to sand fly fever Sicilian virus, 80 and 76% respectively. A much lower rate was found in those at the service academy in Sargodha, in part because of the much lower rate in students. Antibody to sand fly fever virus, Naples and Sicilian, was present in 23% and 26% of 92 students 16-19 years of age, respectively, compared with 55% and 73% of 38 staff and faculty >19 years of age ($P < 0.001$ both comparisons). An increase in antibody to sand fly fever viruses with age was also suggested in patients with febrile illnesses, but this was not statistically significant, perhaps because of the small number of patients ≤19 years of age. Antibody of the IgM class against sand fly fever, Naples or Sicilian strains, in titers of ≥1:200 was detected in 5 and 10%, respectively, of subjects from the service academy, 3 and 4% of recruits at Mardan, and 2 and 1% of patients with febrile illnesses. No age predilection was evident.

Evidence of infection with flaviviruses, in particular West Nile virus, was seen in

37-46% of the three groups (Figure 1). The prevalence increased with age. In those from the service academy, 38% of 91 ≤19 years had antibody to West Nile virus compared with 82% of 38 ≥20 years of age (P= 0.00002). The prevalence of antibody to West Nile virus in patients with febrile illnesses was 46%. Again, a trend toward a higher prevalence in those ≥20 years of age was noted but was not statistically significant. A serologic reaction to another flavivirus, Japanese B encephalitis virus, was also seen in 116 (32%) of subjects from Sargodha and 83 (35%) of patients with febrile illnesses. However, in only 3 (3%) of subjects from Sargodha and 9 (11%) of patients with febrile illnesses, were titers higher against Japanese B than West Nile suggesting cross reactions between these antigenically related viruses. None of the subjects from the service academy had evidence of antibody to Crimean-Congo hemorrhagic fever virus.

DISCUSSION

This study demonstrates that a number of agents that cause febrile illnesses are prevalent in Pakistan and may be important causes of illness in travelers or others visiting the country. Leptospirosis is present in Pakistan, with reservoirs in rodents, dogs, cattle, buffalo and bandicoots \5/. Patients hospitalized in Karachi had a seroprevalence of 25% (14/56), with reactions to *Leptospira icterohemorrhagiae* in 6 patients and *L. grippotyphosa* in eight \5/. In neighboring Afghanistan, 17 (1.4%) of 1,214 sera were positive by the macro slide agglutination test for leptospirosis \11/. This low prevalence is consistent with findings in two of our populations. However, in military recruits, the finding of significant titers of IgM in 16%, including 11% in whom a four fold rise in titers was detected, suggests that some of the illnesses experienced in these military recruits were acute leptospirosis

Sand fly fever is usually a relatively mild disease, with 2-4 days of fever; however, the post-febrile period may be marked by a feeling of weakness, lassitude and depression of up to two weeks \1/. Because of the potential for debility in large numbers of non-immune hosts, sand fly fever has long been a disease of military importance \1/. The disease seems to be more prevalent in central and western portions of Pakistan. Studying sand fly fever in the Khyber Pass area of Pakistan-Afghanistan and in the plains southeast of Teheran, Iran in 1959, workers from the Walter Reed Army Institute of Research and the University of Maryland isolated 8 sand fly fever viruses from the plasma of patients with febrile illnesses \12/. Sandflies collected in the same areas yielded isolates of sand fly fever virus in one-third of the pools of sand flies \12/. Some were of the Sicilian type, while the others were neither Sicilian nor Naples. In a study by the Naval Medical Research Unit in Cairo (NAMRU-3), only one of 43 sera from patients from eastern and southern Pakistan reacted with sand fly fever virus Sicilian, and none reacted with Naples, Karimabad or Salehabad \13/. Serologic studies of patients in Karachi, Pakistan revealed 2.7% of 75 sera had a positive neutralization test for sand fly fever, Sicilian, while 9.3% were positive for sand fly fever, Naples \14/. Antibodies to sand fly fever were detected in only 7 (0.6%) of 1214 sera tested from 4 widely separated villages in Afghanistan \11/. Antibody prevalence to sand fly fever, Naples and Sicilian, in Iran was found in 17 and 25%, respectively, but antibody to another strain, Karimabad, was much higher (66%) \15/. Antibody to Karimabad was not found in sera from Pakistan \13, 14/. The prevalence of antibody increases rapidly with age \15/.

Since no vaccine or specific treatment is available, protective clothing, insect repellents and very fine mesh bed nets are all important personal protective measures. Leishmania, which share with the sand fly fever viruses the sand fly as a vector and the

gerbil as a host , could also be prevented by these personal protection measures. Ribavirin, a broad spectrum anti-viral agent, has been shown to protect against viral challenge in animals \16/.

West Nile virus usually causes a mild disease in children but may cause severe disease, including encephalitis, in adults. West Nile virus was isolated from plasma of 2 of 173 patients with febrile illnesses in 1964 in Rawalpindi \17,18/. Five additional isolates were obtained from 144 patients with fever in Lahore in 1965 \19/. The clinical course of these patients was characterized by fever, severe headache, retrobulbar pain, myalgias, lymphadenopathy and leukopenia. Rash was present in some patients \19/.

Seroprevalence studies have revealed neutralizing antibody to West Nile virus in 33% of residents in a semi-arid area in Punjab Province and in 38% in the Changa Manga National Forest, also in Punjab Province \20/, while in Karachi, the seroprevalence was 50% \21/. All three surveys found an increasing prevalence with age. Only 5 (11.6%) of human sera from Pakistan had antibody when tested by the NAMRU-2 \22/. In the mountains of northern Pakistan, no antibody was detected in a survey of 93 people \19/. West Nile virus has been isolated from *Culex* mosquitos near Rawalpindi \18/ and Lahore \23/. Serologic evidence of West Nile virus was found in about one-third of all birds and buffalos studied near Lahore \20/. In Afghanistan, only one of four villages had an appreciable rate of antibody to West Nile virus \11/. In one village located in the southwestern portion of Afghanistan along a river, 255 of 263 (97%) residents had antibody to West Nile by the hemaglutination inhibition test. Antibody was uniformly present in all but 2 subjects 3 years of age and older. The seroprevalence of West Nile virus neutralizing antibodies in 13 communities in Iran ranged from 0-96%, depending on the region \24/.

Even though over 30% of the study subjects had antibody reactive with Japanese B

encephalitis (JE) virus, it is unlikely that JE is prevalent in Pakistan. Nearly all sera had higher titers against West Nile virus. This cross-reactive phenomenon has been noted by others \11, 21, 23/. In 17 subjects that had equal titers against both West Nile and JE, neutralization antibody titers were higher against West Nile\11/, suggesting cross reactivity among these flaviviruses. No viral isolates of JE have been made in Pakistan \19/. The disease is prevalent in parts of India \3, 25, 26/ requiring extensive immunization \3, 27/ but has not been reported in areas near Pakistan \3/. Even though the main vector for JE, *Culex tritaeniorhynchus*, is a very common mosquito throughout the region \19, 23/, domestic swine, which act as amplifying hosts, are proscribed by Islam. The wild boar may represent a reservoir and amplifying host, though it does not ordinarily live near people. Cattle and buffalo, which do live in close proximity to people, are not thought to be good intermediate hosts for JE \25, 28/.

Other flaviviruses that might give a serologic cross reaction with West Nile are not prevalent in Pakistan. Yellow fever is not present in Pakistan, nor was yellow fever vaccine given to those studied. Dengue virus caused epidemics in Calcutta in 1966 and in Delhi, India in 1982 and 1988 \29, 30, 31/ but has only once been isolated from a patient in Pakistan \19/. This was in Lahore, which is near the Indian border. The vector for dengue, *Aedes albopictus,* is present in the area around Lahore \23/. A low prevalence of antibody reactive with dengue has been found in some surveys, but this may represent a cross-reaction with other flaviviruses \21, 22/. Other flaviviruses, such as Russian spring-summer encephalitis virus and Kyasanur Forest disease, occur in neighboring countries but have not been isolated in Pakistan \19, 32/.

Crimean-Congo hemorrhagic fever (CCHF) occurs in Pakistan and has caused at least 50 cases that have reached medical attention since 1976. Infection has occurred in those working with animals, and in two instances, a total of 13 medical staff were infected

when surgical operations were performed on patients thought to have bleeding peptic ulcer disease. In both outbreaks, the surgeon died of nosocomial infection \4, 19, 1987 unpublished report by Burney/. The vector is the tick of the genus *Hyaloma*. Two isolations of virus from ticks were made from the Changa Manga Forest in 1965 \19/. We found no serologic evidence of CCHF in our present surveys. Other serosurveys in Pakistan, a border area in Kashmir State in India, and Iran have also found a very low prevalence of antibody, suggesting a low infection rate or low survival rate in those infected \13, 19, 33/. No isolates had been made in India as of 1986 \33/, but outbreaks have occurred in other countries bordering Pakistan, such as Iraq and the Soviet Union \34/. Avoidance of ticks and infected animals, and universal precautions in health care settings should prevent infection and the life-threating hemorrhagic fever \35/.

While work on leptospirosis, West Nile virus and sand fly fever viruses has been done in southern and eastern Pakistan, this is the only serosurvey of populations in northern Pakistan, which includes the capital city of Islamabad. With many foreign visitors to this area who would be susceptible, the occurrence of febrile illness should bring to mind these possibilities.

Acknowledgment: Dr. Peter L. Perine developed the protocol for Pyrexia and unknown origin used in this study.

REFERENCES

1. Sabin AB, Philip CB, Paul JR, 1944. Phlebotomus (Pappataci or sandfly) Fever, a disease of military importance. J Am Med Assn 125:603-606.

2. Tesh RB. Undifferentiated Arboviral Fevers. In Tropical and Geographic Medicine 2nd Ed. Ed. Warren KS and Mahmoud AAF. McGraw Hill 1990

3. Bu'Lock FA, 1986. Japanese B virus encephalitis in India - a growing problem. Quarterly J Med 60: 825-836.

4. Burney MI, Ghafoor A, Saleen M, Webb PA, Casals J, 1980. Nosocomial Outbreak of Viral Hemorrhagic Fever caused by Crimean Hemorrhagic Fever-Congo Virus in Pakistan, January 1976. Am J Trop Med Hyg 29:941-947.

5. Ahmed IP, 1987. Serological studies on leptospirosis in Pakistan. J Pak Med Assn 37:233-36

6. Ratman S, Subramanian S, Madanagopalan N, Sundararaj T, Jayanthi V, 1983. Isolation of leptospires and demonstration of antibodies in human leptospirosis in Madras, India. Trans Ray Soc Trop Med Hyg 77;455-458.

7. Malik IA, Qureshi MS, Luqman M, Qamar MA, Ahmed A, Legters LJ, Ahmad M, Akhtar MA, 1988. Epidemics of non-A, non-B hepatitis in Pakistan. Tropical Doct 18:99-101.

8. Iqbal M, Ahmed A, Qamar A, Dixon K, Duncan JF, Islam NU, Rauf A, Bryan JP, Malik IA, Legters LJ, 1989. An outbreak of enterically transmitted non-A, non-B hepatitis in Pakistan. Am J Trop Med Hyg 40:438-43.

9. Meegan JM, Yedloutschnig, Peleg BA, Shy J, Peters CJ, 1987. Enzyme-linked immunosorbent assay for detection of antibodies to Rift Valley fever viruses in ovine and bovine sera. Am J Vet Res 48: 1138-1141

10. Brand OM, Allen WP, 1970. Preparation of noninfectious arbovirus antigens. Appl Micro 20:298-302.

11. Buck AA, Anderson RI, Kawata K, Abrahams IW, Ward RA, Sasaki TT, 1972. Health and Disease in rural Afghanistan. The John Hopkins School of Hygiene and Public Health, Baltimore, MD

12. Barnett HC, Suyemoto W, 1961. Field Studies on Sandfly Fever and Kala-Azar in Pakistan, in Iran and in Baltistan (Little Tibet) Kashmir. Trans New York Acad Sci 23:609-617.

13. Darwish MA, Hoogstraal H, Roberts TJ, Ghazi R, Amer T, 1983. A sero-epidemiological survey for Bunyaviridae and certain other arboviruses in Pakistan. Trans R Soc Trop Med Hyg 77:446-450.

14. Tesh RB, Saidi S, Gajdamovic SJ, Rodhain F, Vesenjak-Hirjan J, 1976. Serological studies on the epidemiology of sandfly fever in the old world. Bull World Health Org 54:663-674.

15. Saidi S, Tesh R, Javadian E, Sahabi Z Nadim A, 1977. Studies on the epidemiology of sandfly fever in Iran: II. The prevalence of human and animal infection with five phlebotomus fever virus serotypes in Isfahan Province. Am J Trop med and Hyg 26:288-293.

16. Oldfield EC, Wallace MR, Hyams KC, Yousif AA, Lewis DE, Bourgeois AL. Endemic infectious diseases of the Middle East. Rev inf Dis 1991;13(Suppl 3):S199-S217.

17. Burney MI, 1966. A report on the role of arthropod borne viruses in human diseases in Rawalpindi and Peshawar area I. Pak J Med Res 26: 215-225.

18. Burney MI, Munir AH, 1966. Role of arthropod borne viruses in human diseases in Rawalpindi and Peshawar area: II Isolation of West Nile virus from human blood and culicine mosquitoes in Rawalpindi area. Pak J Med Res 26:271-285.

19. Hayes CC, Burney MI, 1981. Arboviruses of public Health importance Pakistan. J Pak Med Assn 31: 16-26.

20. Hayes CG, Baqar S, Ahmed T, Chowdhry MA and Reisen WK, 1982. West Nile virus in Pakistan. 1. Sero-epidemiological studies in Punjab Province. Trans of the Royal Soc of Trop Med and Hygiene 76:431-436.

21. Sugamata M, Ahmed A, Miura T, Takasu T, Kono R, Ogata T, Kimura-Kuroda J, Yasui K, 1988. Seroepidemiological study of infection with West Nile virus in Karachi,

Pakistan, in 1983 and 1985. J Med Vir 26: 243-247.

22. Darwish MA, Hoogstraal H, Roberts TJ, Ahmed IP, Omar F, 1983. A sero-epidemiological survey for certain arboviruses (Togaviridae) in Pakistan. Trans R Soc Trop Med and Hyg 77:442-445.

23. Reisen WK, Hayes CG, Azra K, Niaz S, Mahmood F, Parveen T, 1982. West Nile virus in Pakistan. II. Entomological studies at Changa Manga National Forest, Punjab Province, 1982. Trans of the Roy Soc of Trop Med and Hyg 76:437-440.

24. Saida S, Tesh R, Javadian E, Nadim A, 1976. The prevalence of human infection with West Nile virus in Iran. Iranian J. Publ Hlth 5:8-13.

25. Umenai T, Krzysko R, Bektimirov TA, Assaad FA, 1985. Japanese encephalitis: Current worldwide status. Bull WHO 63: 625-631.

26. Kumar R, Misra PK, 1988. Japanese encephalitis in India. Indian Pediatr 25:354-60.

27. Rao Bhau LN, Singh G, Gowal D, Saxena SN, Kobayashi M, Oya A, Yoshioka I, 1988. Safety and efficacy of Japanese encephalitis vaccine produced in India. Indian J Med Res 88:301-7.

28. Ilkal MA, Dhanda V, Rao Bu George S, Mishra AC, Prasanna Y, Gopalkrishna S, Pavri KM, 1988. Absence of viremia in cattle after experimental infection with Japanese

encephalitis virus Trans R Soc Trop Med Hyg 82: 628-31.

29. Acharya SK, Buch P, Irshad M, Gandhi BM, Joshi YK, Tandon BN, 1988. Outbreak of dengue-fever in Delhi. Lancet ii:1485-1486.

30. Rao CV, 1987. Dengue fever in India. Ind J Ped 54:11-14.

31. Rao CV, Bagchi SK, Pinto BD, Ilkal MA, Bharadwaj M, Shaikh BH, Dhanda V, Dutta M, Pavri KM, 1985. The 1982 epidemic of dengue fever in Delhi. Indian J Med Res 82: 271-275.

32. Pavri K, 1989. Clinical, clinicopathologic, and hematologic features of Kyasanur Forest disease. Rev Inf Dis 11: S854-S859.

33. Rodrigues FM, Padbidri VS, Ghalsasi GR, Gupta NP, Mandke VB, Pinot BD, Hoon RS, Bapat MB, Rao CVRM, 1986. Prevalence of Crimean haemorrhagic fever-Congo virus in Jammu and Kashmir State. Ind J Med Res 84:134-138.

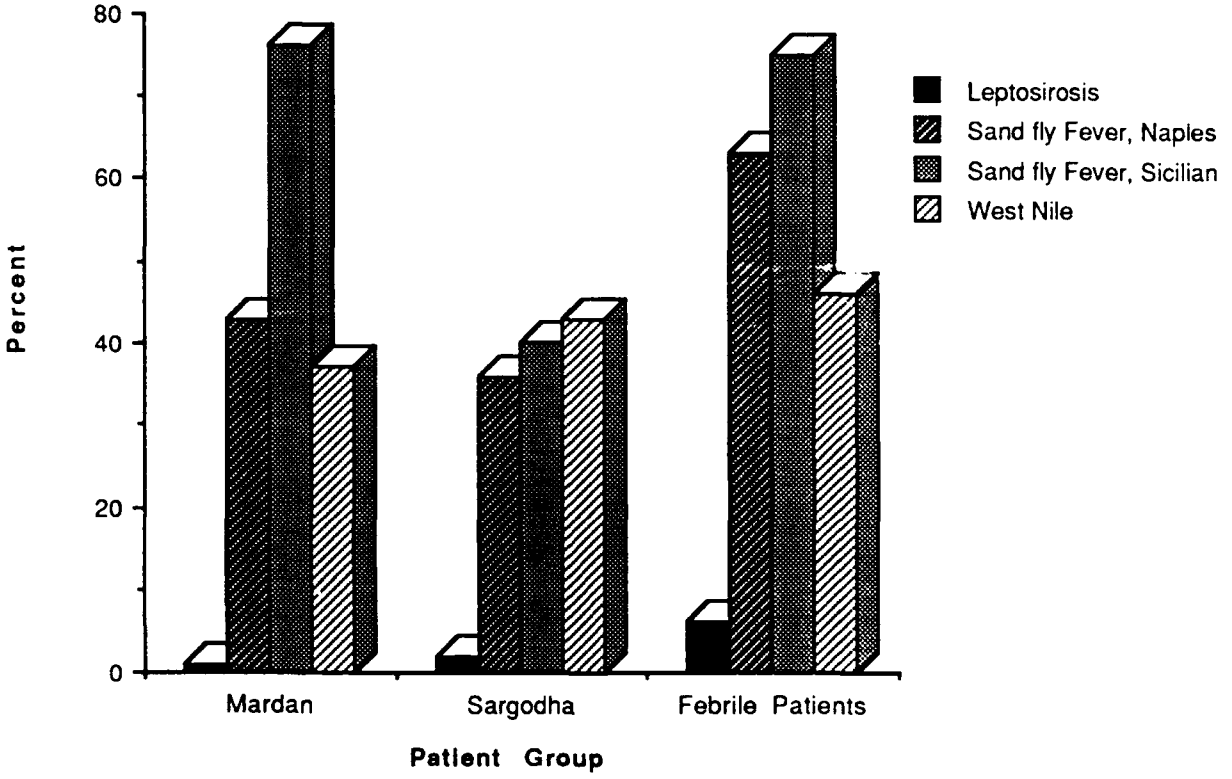
34. Al-Tikriti SK, Al-Ani F, Jurji FJ, Tantawi H, Al-Moslih M, Al-Janabi N, Mahmud MIA, Al-Bana A, Habib H, Al-Munthri, H, Al-Janabi A, Al-Jawahry K, Yonan M, Hassan F, Simpson DIH, 1980. Congo/Crimean haemorrhagic fever in Iraq. Bull World Health Organ 59:85-90.

35. Centers for Disease Control: Management of patients with suspected viral hemorrhagic fevers. MMWR 37:(Suppl)3, 1-16, 1988.

Figure one. Prevalence of antibody to leptospirosis, sand fly fever, Naples and Sicilian, and West Nile virus in recruits at Mardan, cadets at Sargodha and febrile patients in Rawalpindi, Pakistani.

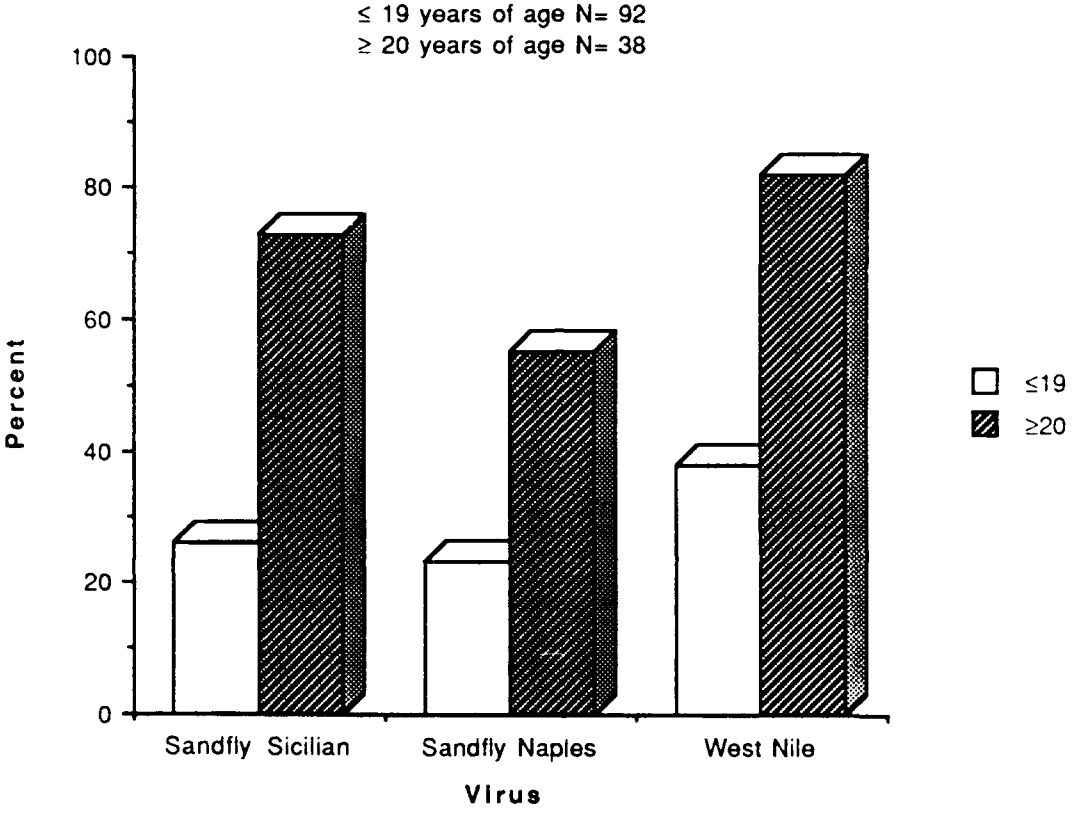
Figure two. Prevalence of antibody to sand fly fever, Naples and Sicilian, and West Nile virus by age in men at an academy in Sargodha, Pakistan.

Figure 1.


80
60
40
20
0
Percent
Mardan
Sargodha
Febrile Patients
Patient Group
Leptosirosis
Sand fly Fever, Naples
Sand fly Fever, Sicilian
West Nile

Prevalence by age of antibody to Sandfly and West Nile Viruses, Sargodha Pakistan

Figure 2



APPENDIX III

Pulse Laboratory

Annual Report for Period Ending December 31, 1990

Title: Etiology of Hepatitis at Military Hospital, Rawalpindi, Pakistan

Investigators: Abdul Rauf, Joe P. Bryan, Aftab Ahmed, Peter L. Perine, L. J. Legters, Iftikhar Malik and others

ABSTRACT

The etiology of hepatitis in 256 men admitted to Military Hospital (MH) in Rawalpindi between December 1989 and November 1990 was studied. During this period, hepatitis B was the etiology in 53 (20.7%) while 203 (79.3%) were non-A, non-B cases. Hepatitis A was not found. Comparison is made with 924 cases of acute hepatitis cases studied in 1987-1989. During that period, hepatitis A was the etiology in 13 (1%), hepatitis B in 264 (29%), and non-A, non-B hepatitis (NANB) in 646 (70%). The proportion of cases of NANB increased from 65% to 77% per year between 1987-90 ($p=0.006$). Between 1987-89, one of 70 patients with HBsAg was positive for anti-delta antibody. All 179 sera tested for IgG anti-HAV were positive, indicating prior hepatitis A infection. Between 1987-89, HBsAg was present in 1/3 of cases of NANB hepatitis while in 1990, 14% of NANB cases had HBsAg indicating convalescent or chronic carriage. The mean age of patients in 1990, 30 years, was similar to previous study years. There is no significant difference in age between patients with HBV and NANB . Evaluation of admission ALT and bilirubin in patients admitted between 1987-89 revealed significantly higher concentrations in patients with hepatitis B than in those with NANB. Patients with HBV seen in 1987-89 were more likely than patients with NANB to have had domiciliary or sexual contact with a patient with hepatitis (12.4% vs 4.2%, $p=0.012$) or to have received injections (38% vs 26%, $p=0.048$). Evaluation of patients in 1990 revealed that patients with hepatitis B were more likely to have had dental treatment compared with patients with non-A, non-B hepatitis (26% vs 7.9%; $p<0.001$).

Hepatitis A, B and agents of NANB (presumably hepatitis C and E) are risks for those living in or visiting Pakistan.

Statement of the Problem

Hepatitis is one of the most common causes of admission to Military Hospital (MH) in Rawalpindi, Pakistan. Initially thought to be largely caused by hepatitis A, serologic studies on sera transported to USUHS and the Walter Reed Army Institute of Research in 1984 showed that only rarely was hepatitis A the cause of acute jaundice in men admitted to MH. Approximately one-third to one-half of the cases were caused by acute hepatitis B, and the others were caused by neither hepatitis A nor B and were termed non-A, non-B (NANB) hepatitis. Almost all patients studied had serologic evidence of prior hepatitis A exposure.

Cases gathered in 1984 from MH by Qureshi, et al. proved to be largely NANB in etiology; one case was caused by hepatitis A, 11 by hepatitis B, but in 38 of 50, neither hepatitis A nor B could be implicated (1). Patients with hepatitis studied at the PULSE between 1984 and 1986 revealed similar findings; 53 of 232 (23%) were caused by hepatitis B, none by hepatitis A, and 179 of 232 (77%) by a NANB agent(s) (2).

Hepatitis C, the major etiology of hepatitis following receipt of blood products, is known to cause disease that often results in chronic hepatitis. However, most of the cases of NANB hepatitis seen at MH had no history of receipt of blood products or intravenous drug use and appeared to have no chronic sequelae of hepatitis.

A new etiologic agent of hepatitis, termed enterically transmitted non-A, non-B hepatitis (ET-NANB), and now also being

called hepatitis E unofficially, was being described in southwest Asia during this time. An outbreak of hepatitis in New Delhi involving 29,000 cases of hepatitis in 1955 and another in 1975-76 in Ahmedabad City, India, which had been thought to be classic outbreaks of hepatitis A, were found not to be caused by hepatitis A or hepatitis B when serologic tests for these viruses became available. Similar outbreaks of NANB hepatitis occurred in the Kashmir, Nepal, the Soviet Union, Africa and Mexico (3), (4), (5). Outbreaks of this type were characterized by exposure to fecally contaminated drinking water (6), clinically apparent disease in young adults, a high case-fatality rate in pregnant women, especially in the third trimester (7, 8), and low secondary attack rates in exposed household members (9).

In order to determine the etiology and epidemiology of hepatitis in men admitted to MH, we began a prospective study of all cases of acute jaundice in February 1987. This report summarizes the serologic and biochemical findings of cases collected through November 1990.

Methods:

Patients: Men at least 16 years of age admitted to MH with jaundice were asked to participate in the study. Investigators obtained informed consent, reviewed the medical record, interviewed the patient with a standardized questionnaire, and performed a physical examination. Laboratory data, including biochemistry and hematology data, were recorded on admission and weekly.

Some cases were missed during May and June of 1987 when the

study was interrupted. The decrease in cases in April, May and June 1990 is unexplained. Cases enrolled in a case-control study of hepatitis in 1989 were evaluated using a different patient questionnaire; however, these cases are counted in the monthly totals.

Laboratory: Sera were divided into aliquots at the PULSE; an aliquot was stored and another was examined by ELISA for hepatitis A and B serologic markers. Commercially available serologic tests were used to study sera for antibody to hepatitis A (HAVAB-EIA), IgM anti-HAV (HAVAB-M EIA), hepatitis B surface antigen (AUSZYME), anti-HBc (CORZYME) and IgM anti-HBc (CORZYME-M) (Abbott Diagnostics). Sera with hepatitis B surface antigen were examined for anti-Delta antibodies (Abbott ANTI-DELTA EIA).

Acute hepatitis B was defined by the presence of IgM anti-HBc, acute hepatitis A by the presence of IgM anti-HAV, and NANB hepatitis by the absence of both these markers. Non-A, non-B cases were further divided by the presence or absence of HBsAg, for the purpose of defining the characteristics of the NANB cases with and without HBsAg.

Total bilirubin, alanine aminotransferase (ALT), aspartic transferase (AST) and alkaline phosphatase were performed at the PULSE using the Centrifichem. Analysis of biochemical data was done on patients in the surveillance study (not in the case-control study) who were males ≥16 years of age admitted in 1987-89, with recorded IgM anti-HBc and IgM anti-HAV, and ALT values, if recorded, above the upper limits of normal for the Centrifichem (1-22 units/liter)

Information on cases studied in 1990 was provided by written communication from Maj General Iftikhar Malik, February 1991. Analysis of data from 1987-1989 was done by Joe P. Bryan, MD,; LCDR, MC, USNR, based on data provided as of August 1990.

Results:

Between December 1989 and November 1990, 256 patients with jaundice were studied. No case of hepatitis A was found in this time. Hepatitis B was the etiology in 53 (20.7%) and 203 (79.3%) were of non-A, non-B etiology. Serologic evidence of hepatitis B infection was found in 100 (49.3%) of the NANB cases, including at least 28 cases with HBsAg.

A comparison of risk factors for hepatitis B and non-A, non-B in patients in this study period are seen in table 1. Dental treatment appeared to be significantly more common in those with hepatitis B than with non-A, non-B hepatitis. No statistical differences in other risk factors were found.

Between February 1977 and November 1991, 1129 patients have been enrolled in the hepatitis surveillance protocol or case-control study. Figure one shows the total number of cases, cases of hepatitis B, and NANB cases by month. Only 13 cases of hepatitis A were diagnosed during this time (not shown on the figure). No definite seasonal trend is noted. Figure 1 and Table 2 indicate that the proportion of cases of hepatitis B and NANB remained relatively constant until 1989, when the proportion of cases of NANB hepatitis increased. The proportion of cases due to NANB hepatitis increased significantly, from 65% in 1987 to 77% in 1989 and 1990 ($p=0.006$, X^2 for trend).

Table 2 shows the number of cases enrolled by etiology and by year for 1987-1990. Overall, 71% of the 1129 cases had a NANB etiology and 28% were caused by hepatitis B. HBsAg was detected in approximately one-third of NANB. During this time, 70 sera with HBsAg were tested for anti-Delta antibody; one was positive.

Additional analysis of biochemical findings on patients for whom serologic and at least partial biochemistries were available. 731 male patients of ≥ 16 years of age were available for study. Fifty-seven patients were excluded because they had normal ALT values including 48 patients without serologic markers for acute hepatitis A or B, and 200 more had no ALT values recorded leaving 474 evaluable patients. Table 4 shows results of these 474 patients who met these criteria. The mean age of those with hepatitis was almost 32 years (range 16-80). The mean age of those with NANB hepatitis was similar to those with hepatitis B and hepatitis A. Patients with NANB hepatitis with HBsAg were similar in age to those who did not have HBsAg.

In this group of patients, hepatitis A was the etiology in only 5 patients in this subset of patients. All 179 patients studied had IgG antibody to hepatitis A indicating previous hepatitis A exposure. Hepatitis B was a more common etiology of hepatitis in this group of patients, causing 31% of the cases of acute hepatitis. In addition to these cases of acute hepatitis B, 142 of 287 (49%) of the cases of NANB hepatitis had antibody to hepatitis B core antigen (anti-HBc), indicating previous exposure to hepatitis B. Altogether, 298/452 (66%) of jaundiced patients studied had evidence of past or present hepatitis B infection.

Evaluation of the biochemical aspects of hepatitis caused by the

various etiologies is also shown in Table 3. Cases of hepatitis B had significantly higher mean levels of total bilirubin (10.4 ± 0.8 vs. 8.3 ± 0.6; mean ± S.E.M.) and ALT (480 ± 65 vs. 263 ± 16) than cases of NANB hepatitis. The mean total bilirubin for cases of NANB hepatitis with HBsAg is similar to those without HBsAg. No difference is noted in the alkaline phosphatase for the HBV and NANB groups.

Risk factors for hepatitis in these patients are shown on Table 3. Comparison is made between patients with IgM anti-HBc and patients without IgM-anti-HBc or anti-HBc; i.e., patients with NANB hepatitis who were susceptible to hepatitis B. Contact with a person with hepatitis was significantly more common in patients with hepatitis B than with NANB. A history of transfusion was significantly more common in those with NANB hepatitis, suggesting that a parenteral route of infection was possible in these nine cases. Surgical operations within the 12 months before onset of illness were a risk factor in only 15 patients. However, a history of injections was present in approximately one-third of each group. Information about dental procedures was not provided for this group of patients.

Similar results are obtained when all cases of NANB hepatitis are considered without excluding those who have serologic evidence of previous hepatitis B. In patients with NANB hepatitis, a history of contact with jaundiced persons was present in 11 of 246 (4.2%); blood transfusions in the last 12 months, 10 of 274 (3.6%); surgical operation in 12 months before admission, 16 of 269 (5.6%); and injections in 12 months before admission, 82 of 202 (28%). Comparing all NANB patients with those who had hepatitis B,

injections (p=0.048) and contact with hepatitis (p=0.003) are statistically significant greater risks for hepatitis B, while transfusions in this group did not appear to be a greater risk factor for NANB hepatitis than for hepatitis B (p=0.11).

Receipt of blood transfusions does not appear to be a major risk factor for hepatitis B infection in this particular population. In fact, a greater proportion of those without serologic evidence of hepatitis B had received blood transfusions, 9 of 149 (6.0%), than those with serologic evidence of hepatitis B, 7 of 293 (2.4%) (Odds Ratio 0.4, p=0.06).

CONCLUSIONS

This four-year prospective study of hepatitis in men admitted to MH indicates that viral hepatitis of both hepatitis B and NANB etiologies continues to be a major cause of morbidity in troops stationed in or near Rawalpindi, Pakistan. The cases of NANB hepatitis may be primarily caused by hepatitis E (HEV; enterically transmitted NANB hepatitis). Several outbreaks of NANB hepatitis in the Pakistan military have been associated with fecally contaminated water (10), (11) which is consistent with findings in outbreaks in other parts of the world (9). Viral particles in stool that react with sera from HEV cases from Africa, India, Nepal and Mexico have been found in cases from Pakistan (9, 12, 13) and in patients during an outbreak of hepatitis at the Air Force Academy in Sargodha, Pakistan, (J. Ticehurst manuscript in preparation). Since no known outbreaks of HEV are included in this four-year hospital surveillance, HEV may be a major cause of sporadic hepatitis morbidity, as well as the major cause of epidemic hepatitis in

Pakistan.

No serologic test is yet readily available for the diagnosis of HEV. Time, labor and material-intensive immuno-electron microscopy is presently the most reliable test. The difficulty of recovering sufficient viral particles from stool of humans has been partially overcome by experimentally infecting cynomolgus and macaques and collecting virus directly from their bile before it reaches the intestine, where the virus apparently is degraded (9). Using this viral source, sufficient virus has been obtained to sequence the genome of the virus which has been partially cloned (14). Using either naturally acquired or recombinant viral antigen, it is hoped that sero-diagnostic tests for HEV will soon be available for testing.

Rapid degradation of HEV in the intestine may account for the low secondary attack rates seen in epidemics of HEV and the low rate of a history of contact with hepatitis cases in our study patients. This suggest that the physical conditions which permit survival of HEV in water or sewage in concentrations large enough to cause symptomatic human infe\ on are narrow and/or infrequently encountered.

Hepatitis C (HCV) may also be a cause of some of the cases of NANB hepatitis in these Pakistani soldiers. Only about 6% of cases of NANB had a history of transfusions in the year prior to admission, but approximately 30% of NANB cases had received injections during this period. Among cases of hepatitis B in a case-control study of hepatitis in 1989, anonymous questioning revealed that approximately 17% had used illicit drugs, 21% admitted sharing needles, and 25% acknowledged homosexual activity within the 6

months prior to admission (authors' unpublished data). Therefore, some patients with NANB hepatitis have risk factors for HCV as well as hepatitis B.

One problem in the diagnosis of HCV is that specific antibody may not be detectable for up to one year after infection. No long-term follow-up of our patients has been done to determine whether chronic hepatitis is present as a sequela or if antibody has developed to hepatitis C.

The next requirements to complete this study of the etiology of hepatitis in Pakistani men include testing sera for the presence of anti-HCV and also for anti-HEV when serologic tests are developed. Patients with HBsAg should be tested for anti-Delta antibody. Patients thought to be chronic carriers of hepatitis B could be tested for hepatitis B e antigen to determine infectivity. Missing information (especially laboratory values for ALT, AST, T. bilirubin etc.) should also be added to the database. Incidence of disease should be calculated based on the numbers at risk. The number of deaths from hepatitis and related causes should also be recorded.

Future research questions, in addition to defining the etiology of hepatitis with better diagnostic tests for hepatitis C and E, should include the evaluation of any candidate vaccines for HEV and the prophylaxis of HEV disease by immune serum globulin which could be prepared from convalescent cases. Vaccine development will depend on the availability of sufficient HEV antigen prepared by recombinant methods.

Specific actions required at present include further investigation into sterilization procedures for dental instruments and the

enforcement of the use of sterile, non-reusable syringes to decrease the propagation of hepatitis B (15). Dentists should also be checked for HBsAg and HBeAg and if found to be infected, be required to wear masks and gloves when providing care. Those in close contact with hepatitis B patients should be immunized with hepatitis B vaccine or at least receive immune serum globulin. Education should also be given about high risk behaviors and means of transmission of hepatitis B.

References

1. Qureshi M, Ahmad M, Khan F, Mushtaq S, Ahmed S. Acute sporadic viral hepatitits: A seromarker study in 50 consecutive cases. J Pak Med Assn 1987;37:231-233.

2. Malik I, Luqman M, Ahmed A, Khan A, Legters L. Sporadic non-A, non-B hepatitis: A sero-epidemiological study in urban population. J Pak Med Assn 1987;37:190-192.

3. Gust I, Purcell R. Report of a workshop: Waterborne non-A, non-B hepatitis. J Inf Dis 1987;156:630-35.

4. Nouasria B, Trepo C, Larouze B, Saimot G, Aouati A. Non-A, non-B acute hepatitis in eastern Algerian adults. Trans Roy Soc Trop Med Hyg 1984;78:137-38.

5. Purcell R, Ticehurst J. Enterically transitted non-A, non-B hepatitis: Epidemiology and clinical characterictics. In: Viral Hepatitis and Liver Disease. Alan R. Liss, Inc, 1988: 131-137.

6. Nouasria B, Larouze B, Dazza M, Gaudebout C, Saimot A, Aouati A. Direct evidence that non-A, non-B hepatitis is a water-borne disease. Lancet 1984;ii:94.

7. CDC. Enterically transmitted non-A, non-B hepatitis in East Africa. MMWR 1987;36:241-44.

8. CDC. Update:Health and nutrition profile of refugees-Ethiopia, 1989-90. MMWR 1990;39:707-718.

9. Bradley D. Enterically-transmitted non-A, non-B hepatitis. Brit Med Bull 1990;46:442-461.

10. Malik A, Qureshi M, Luqman M, Qamar M, Ahmed A, Legters L, Ahmad M, Akhtar M. Epidemics of non-A, non-B hepatitis in Pakistan. Trop Doc 1988;17:99-101.

11. Iqbal M, Ahmed A, Qamar A, Dixon K, Duncan J, Islam N, Rauf A, Bryan J, Malik I, Legters L. An outbreak of enterically transmitted non-A, non-B hepatitits in Pakistan. Am J Trop Med Hyg 1989;40:438-443.

12. Arankalle V, Ticehurst J, Sreenivasan M, Kapikian A, Popper H, Pavri K, Purcell R. Aetiological association of a virus-like particle with enterically transmitted non-A, non-B hepatitis. Lancet 1988;i:550-554.

13. DeCock K, Bradley D, Sandford N, Govindarajan S, Maynard J, Redeker A. Epidemic non-A, non-B hepatitis in patients from Pakistan. Ann Int Med 1987;106:227-30.

14. Reyes G, Purdy M, Kim J, Luc K-C, Young L, Fry K, Bradley D. Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. Science 1990;247:1335-1339.

15. CDC. Guidelines for prevention of transmission of Human Immunodeficiency virus and hepatitis B virus to health-care and public-safety workers. MMWR 1989;38(no. S-6).

TABLE I

RISK FACTORS FOR ACUTE HEPATITIS B AT MH, 1990

VARIABLE	HEPATITIS B	NON-A, NON-B	P VALUE
CONTACT WITH HEPATITIS	4/53(7.5)	22/203(10.8)	0.48
PREVIOUS HISTORY OF JAUNDICE	2/53(3.8)	19/203(9.4)	0.19
TRANSFUSION IN 6 MONTHS BEFORE ADMISSION	2/53(3.8)	6/203(3.0)	0.76
SURGERY IN 6 MONTHS BEFORE ADMISSION	1/53(2.0)	10/203(5.0)	0.33
INJECTIONS IN 6 MONTHS BEFORE ADMISSION	3/53(5.6)	26/203(12.8)	0.14
DENTAL TREATMENT	14/53(26)	16/203(7.9)	0.0002

Table 2. Hepatitis at Military Hospital, 1987-1990

Year	NANB without HBsAg	NANB with HBsAg	Total NANB	IgM ANTI-HBc POS	IgM ANTI-HAV POS	DELTA POS	TOTAL/YEAR
1987	123	69	192	93	9	1	295
	42%	23%	65%	32%	3%		
1988	120	81	203	98	4	0	305
	39%	27%	67%	32%	1%		
1989	89	63	251	73	0	0	324
incomplete HBsag data	27%	19%	77%	23%			
1990 (excluding Dec)			158	47	0	0	205
			77%	23%	0%	0%	
TOTAL	332	213	804	311	13	1	1129
% OF TOTAL 1987-1990	29%	19%	71%	28%	1%	0%	

Table 3.

Risk factors for acute Hepatitis B Compared with Patients with Non-A, non-B hepatitis who are susceptible to hepatitis B Military Hospital, Rawalpindi 1987-1989

Variable	Hepatitis B	Non-A, Non B anti-HBc neg	p value
Contact with patient with hepatitis	18/145 (12.4%)	5/134 (4.2%)	0.012
Transfusion in 12 m before admit	1/160 (0.6%)	8/136 (5.8%)	0.02
Surgery in 12 months before admit	9/160 (5.6%)	9/146 (6.2)	0.84
Injections in 12 months before admit	51/156 (39%)	45/147 (30)	0.12
History of previous jaundice	15/159 (10.5%)	16/147 (10.7%)	N.S.

Table 4.

Biochemical findings in 474 men ≥ 16 years of age in hepatitis protocol with abnormal ALT*

	All Non-A, non-B	NANB without HBsAg	NANB with HBsAg	Hepatitis B	Hepatitis A	All Hepatitis
Number in Study						
N=	287	179	108	161*	5	474
Age						
M ± S.D.	32 ± 11	32 ± 11	32 ± 12	30 ± 10	26.1 ± 5	31.6 ± 10.8
Total Bilirubin						
N=	121	85	33	74	2	206
M ± S.E.	8.3 ± 0.6	8.5 ± 0.8	7.1 ± 0.9	10.4 ± 0.8	15.3 ± 3.6	9.3 ± 0.5
Alanine Amino Transferase						
N=	287	179	108	163	5	474
M ± S.E.	263 ± 16	253 ± 20	266 ± 28	480 ± 65	416 ± 107	338 ± 25
Alkaline Phosphatase						
N=	261	155	102	151	4	419
M ± S.E.	131 ± 5.4	134 ± 7.5	122 ± 7.3	135 ± 5.6	171 ± 49.4	133 ± 3.9

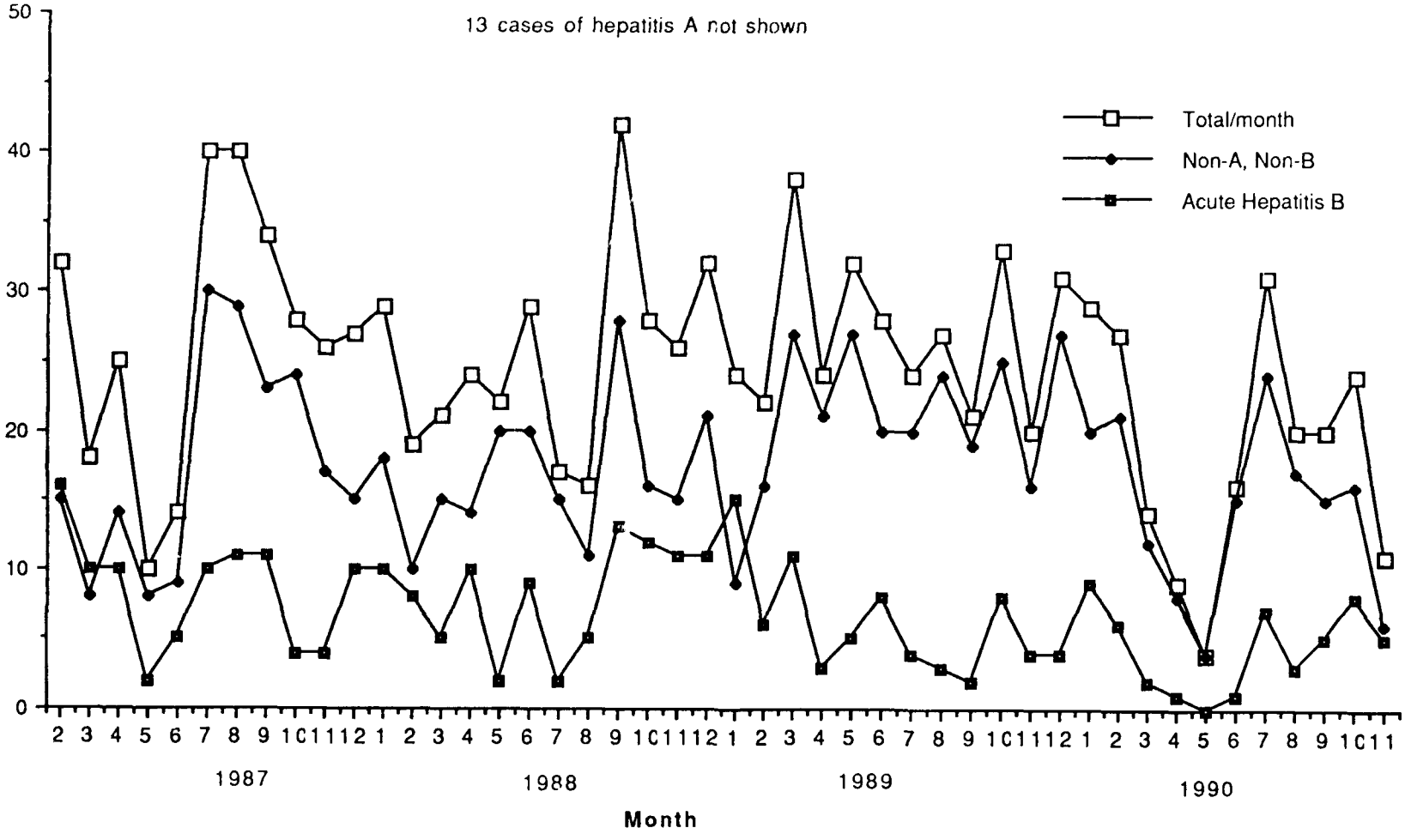
* includes one with IgM anti-HBc and anti-HAV

Figure 1.

Hepatitis at Military Hospital, 1987-1990

N=1116

13 cases of hepatitis A not shown



APPENDIX IV

PULSE LABORATORY

Annual Report for period ending December 31, 1990

Title: Case Control Study of Risk factors for hepatitis B in Pakistani men

Investigors:

Data analyzed and report written by Joe P. Bryan

Study designed and conducted by Mohammed Iqbal, Abdul Rauf, Kenneth Dixon, Aftab Ahmed, Iftikhar A. Malik, and Llewellyn J. Legters

Statement of the problem

Hepatitis is one of the most common causes for admission of men to Military Hospital in Rawalpindi. Hepatitis B accounts for approximately 100 cases/year or approximately one-third of all cases of hepatitis in men (authors' unpublished data). This proportion appears to have been relatively stable since 1984. Known risk factors for hepatitis B transmission in the U.S. military include illicit use of parenteral drugs, household contact with hepatitis B carriers, multiple heterosexual partners and body fluid exposure as a health care worker (1-4). In addition, homosexual activity in males is a well known risk factor for hepatitis B (5, 6). Nosocomial infections of hepatitis B have occurred from contaminated vaccines (7), contaminated solutions for intravenous injection (8), and after surgical or dental procedures (9). Risk factors for hepatitis B have not been readily apparent in patients in Pakistan. Since some of these risk factors may be of a sensitive nature for military personnel in Pakistan, a case-control study with a randomized response questionnaire was conducted.

METHODS

Patient selection

Four hundred patients, 200 with jaundice and 200 with diseases thought to be unrelated to hepatitis, who were willing to be interviewed about risk factors for hepatitis B were enrolled in this study between 14 December 1988 and 11 January 1990. Of these,

there is no information about the diagnosis in the database for 22 cases: 47, 51, 52, 59, 66, 112, 128, 130, 156, 177, 195, 234, 241, 242, 248, 244, 253, 254, 256, 257, 301 and 314.

Administration of the questionnaire

A questionnaire with a number of questions about possible risk factors for hepatitis B was devised. In order to provide confidentiality and a degree of truthfulness in response to some sensitive questions, patients were interviewed individually in a private room. The patient's response to each question was guided by the use of a "Randomized Response Device" (10). This device consists of a box containing a six-sided die which is tossed by the patient and seen only by him. After the interviewer asks a question, the patient tosses the die. The proper response to the question is automatically "yes" if the die shows 1 and "no" if the die shows 2. If the die shows 3, 4, 5, or 6, the patient was to answer the question truthfully. The patient's response was recorded by the interviewer.

In order to estimate the proportion of "yes" answers which were answered after an instruction to answer correctly, the expected number of automatic "yes" and "no" answers (die equals 1 or 2) must be factored out (10). This proportion is expressed as:

p = number of yes answers - 1/6 N divided by 4/6 N

where N = number of times die is cast (or number of patients).

Therefore, approximately 17% of the responses should be "yes" automatically and for questions with less than this response rate, the random response nature of the study must be questioned.

Laboratory evaluation

Serologic tests for hepatitis B (anti-HBc, IgM-anti-HBc, HBsAg) and acute hepatitis A (IgM-anti-HAV) were performed on all cases and controls using commercially available ELISA test kits (Abbott Diagnostics, Abbott Park, Ill.). Acute hepatitis B is defined by the presence of IgM anti-HBc, acute hepatitis A by the presence of IgM anti-HAV and non-A, non-B hepatitis was the diagnosis in patients with neither marker.

Analysis of results

The responses to individual questions by patients with hepatitis B are compared with sex-matched non-hepatitis controls who were susceptible to hepatitis B: i.e., negative for anti-HBc (assuming none has been immunized for hepatitis B). Since confidence intervals of the estimated proportions are so large because of the nature of the randomized response, direct comparison between cases and controls is not statistically warranted .

Note: For many cases, biochemical data is not provided. Serologic studies for hepatitis B are not complete for case numbers 88, 305, and 112. The following cases are labeled "hepatitis cases," but signs of hyperbilirubinemia, such as jaundice, scleral icterus, dark urine or light stools were reportedly absent and biochemistries were not

provided: 138, 153, 160, 197, 198, 199, 212, 299 and 260. These cases are included in the present analysis, but confirmation with biochemical evidence of hepatitis should be present before these cases are included in any final analysis.

RESULTS

Table 1 shows the serologic evaluation of cases and controls who were entered into the study and who were evaluable. Forty-three cases of hepatitis met the criteria for acute hepatitis B. Four patients in the control group had detectable IgM anti-HBc and were disqualified from further analysis. An additional 69 (37%) controls had evidence of prior hepatitis B infection and were disqualified because they were not susceptible to hepatitis B. The proportion of hepatitis cases and controls who appeared to be convalescent or chronic carriers of hepatitis B was similar in each group, 19% and 20% respectively.

Forty-three cases of acute hepatitis B and 114 controls were therefore available for comparison (Table 2). Case and control groups were well matched with regard to age, marital status and socioeconomic class. Information about rank and duration of military service was not provided.

Risk factors for hepatitis B transmission, present in cases in somewhat higher proportion than in controls after correction for automatic answers, include a history of immunizations, intramuscular or intravenous injections, receipt of antibiotics (often given IM), extramarital sex, multiple sexual partners, use of intravenous drugs,

and a history of venereal diseases or genital lesions. Homosexual activity, including multiple homosexual partners, appeared to be more common in controls than in hepatitis patients. Fewer than expected (based on random chance) cases or controls had medical exposures such as surgical operations, blood transfusions, dental treatments, barber shaving, a history of tattoos, use of drugs or sharing of needles in last 6 months, indicating the response to these questions may not have been random.

DISCUSSION

Based on the above findings, risk factors for hepatitis B in these Pakistani men are similar to those in western countries. Reusable syringes and needles which are autoclaved may be a source for transmission of hepatitis B, since hepatitis B is more resistant to heat than other viruses. Dental treatment did not appear to be a significant risk factor for hepatitis B in this study. However, it was significantly more common in patients with hepatitis B than in patients with non-A, non-B hepatitis at MH in 1990 (authors' unpublished results). Sexual transmission may have occurred in some men.

Four patients in the control group had IgM anti-HBc. In addition, the fact that 38% of controls had evidence of exposure to hepatitis B (presence of anti-HBc) and 20% had HBsAg, prevalences similar to those is in the total hepatitis group, suggests that hepatitis B transmission is ongoing in both groups. Without intervention, many who are not presently infected will be infected in the future. The

fact that in the control group, 13% (after correction) had homosexual activity in the previous six months and 4% (after correction) had sex with > 5 men in the previous six months suggests that these men may be at high risk for hepatitis B.

Specific recommendations to decrease risk factors for hepatitis B are: 1) to insure that medications and immunizations are given with sterile, non-reusable syringes, 2) education about risk factors for hepatitis B infection should be given in an attempt to modify behavior, and 3) education should be given about acute and chronic sequelae of infection, including cirrhosis and hepatic carcinoma. Immunization of those susceptible to hepatitis is an expensive but possible method for interruption of transmission.

Requirements to complete this study include the coding of the 22 patients who are not defined as either a case or control and the completion of hepatitis serologic marker and biochemical data. The study would have greater power if a greater number of cases of hepatitis B were enrolled. In the present study, because only 43 cases of acute hepatitis B were enrolled and 1/3 of these had to be discarded from analysis because of the automatic nature of their responses, only 29 patients are actually evaluable. Addition of cases could be done more efficiently by performing the hepatitis serologic markers soon after admission so that cases with hepatitis B could be specifically sought.

Table 1.

Results of evaluable cases

	Hepatitis		Controls	
	No.	%	No.	%
patients	190		188	
IgM anti-HAV	0	0	0	0
IgM anti-HBc	43	22	4	2
anti-HBc without IgM anti-HBc	55	29	69	37
HBsAg without IgM anti-HBc	37	19	34	20

Table 2.

Comparison of risk factors for hepatitis B

	Hepatitis B		Controls	
	IgM anti-HBc No. answering	(%)* yes	anti-HBc neg No. answering	(%)* yes
Patients	43		114	
Mean age	28		26	
Marital status single	13	(30)	51	(45)
Socioeconomic class 2	43	(100)	51	(98)
Contact with hepatitis patient	4	(9)	3	(2)
Jaundice in spouse, children or other	0		0	
History of blood products	0		3	(3)
Local surgery	0		0	
General surgery	0		2	(2)
History of immunizations	13	(21)*	26	(9)*
Self shaving	34	(93)*	91	(96)*
Barber shaving	2	(5)	8	(7)
Dental treatment (6 months)	2	(5)	(9)	(8)
History of IM or IV injections	9	(7)*	16	(0)*
History of antibiotics	8	(3)*	12	(0)*
History of tattoo	1	(2)	5	(4)
Sex with other than wife	8	(3)*	16	(0)*
Sex with > 5 women (6 months)	10	(10)*	21	(3)*
Sex with man (6 months)	7	(0)*	29	(13)*

Sex with > 5 men (6 months)	3	(7)	22	(19)
Use of Drugs	4	(9)	18	(16)
Inject drugs (6 months)	9	(7)*	19	(0)*
Share needles (6 months)	3	(7)	24	(21)
Treatment for VD (6 months)	10	(10)*	20	(1)*
Urethral Discharge (6 months)	7	(0)*	25	(8)*
Genital lesions (6 months)	7	(0)*	26	(9)*

*Indicates corrected proportion based on formula in Methods. Corrections are done only on parameters which appear to have been answered according to randomized response; i.e., at least 17% answered yes.

References

1. Bancroft W, Takafuji E. The military and hepatitis B. Vaccine 1990;8s:s33-36.

2. Hyams K, Palinkas L, Burr R. Viral hepatitis in the US Navy, 1975-84. Am J Epidemiology 1989;130:319-326.

3. Lemon S, Lednar W, Bancroft W, Cannon H, Benenson M, Park J, Chruchill F, Tezak R, Erdtmann F, Kerchdoerfer F, Lewis P, James J, Miller R. Etiology of viral hepatitis in American Soldiers. Am J Epidemiology 1982;116:438-450.

4. Scott R, Schneider R, Snitbhan R, Karwacki J. Factors relating to transmission of viral hepatitis in the United States military population stationed in Thailand. Am J Epidemiology 1981;113:520-528.

5. Kingley L, Rinaldo D, Lyter D, Valdiserri R, Belle S, Ho M. Sexual transmission efficiency of hepatitis B virus and human immunodefiency virus among homosexual men. JAMA 1990;264:230-234.

6. Alter M, Hadler S, Margolis H, Alexander J, Hu P, Judson F, Mares A, Miller J, Moyer L. The changing epidemiology of hepatitis B in the United States: Need for alternative vaccination strategies. JAMA 1990;263:1218-1222.

7. Seef L, Beebe G, Hoofnagle J, Norman J, Buskell-Bales Z, Waggoner J, Kaplowitz N, Koff R, Petrini J, Schiff E, Shorey J, Stanley M. A serologic follow-up of the 1942 epidemic of post-vaccination hepatitis in the United States Army. N Engl J Med 1987;316:965-970.

8. Oren I, Hersow R, Ben-Poreth E, Krivoy N, Goldstein N, Rishpon S, Shouval D, Hadler S, Alter M, Maynard J, Alroy G. A common-source outbreak of fulminant hepatitis B in a hospital. Ann Int Med 1989;110:691-698.

9. Shaw F, Barrett C, Hamm R, Peare R, Coleman P, Hadler S, Fields H, Maynard J. Lethal outbreak of hepatitis B in a dental practice. JAMA 1986;(255):3260-3264.

10. Comstock G, Conde J, Helsing K. A simple randomized response device. Am J Epi 1985;122:187-190.

APPENDIX V

PULSE LABORATORY

ANNUAL REPORT FOR PERIOD ENDING DECEMBER 31, 1990

Title: Anopheline Vectors of Malaria in Urban and Rural Areas of Pakistan

Investigators: Wahid ur Rehman, Noreen Bukhtiari, Richard Andre, Lance Sholdt, Iftikhar Malik

Statement of the Problem

Malaria is a important public health problem in many developing countries, including Pakistan. While the disease occurs throughout much of the country, its annual incidence varies widely by location and year. Five anopheline species have been incriminated as important vectors of malaria in specific ecological settings within Pakistan. These include *Anopheles culicifacies* (rural), *An. stephensi* (urban), *An. superpictus* (desert), *An. fluviatilis* (hilly regions) and *An. maculatus* (mountain regions)[1].

Intensive studies on the bionomics of *An. culicifacies* and *An. stephensi* have been conducted, and valuable observations have been reported on adult mobility, longevity, survivorship, activity cycles, gonotrophic rhythm, mating behavior, resting behavior, sampling methods and population size [2,3,4,5,6,7]. Regardless, important questions remain as to the vector status of these species and others (such as *An. pulcherrimus*) in specific ecological settings. The role of local anopheline species in the transmission of malaria needs to be quantified for various ecological settings in rural and urban environments of Pakistan. This can be done by identifying the extent of man-vector contact by season of the year, time and location; by determining sporozoite rates by species, location collection method and season of the year, and by quantifying host preferences of different anopheline species. An issue that also requires special attention is the question of which species is transmitting malaria and the particular ecological circumstances under which transmission occurs at high altitudes in the Himalayan region of Pakistan [8]. Finally, there has not been a comprehensive mosquito fauna survey of the country since the 1940's to determine species distribution and abundance, as well as possible introductions of new species. Such an endeavor is needed to develop illustrated keys to anophelines of the region and to establish reliable reference collections for use by US and Pakistani investigators. Answers to these questions require specific and detailed information about host preferences, sporozoite positivity rates under natural conditions, and the taxonomic status of local anopheline species.

This report summarizes the findings to date on the anopheline vectors in Pakistan. Most of the data presented is preliminary information, which is being used for the development of full-scale studies on the subject.

Methods

Using standard methods, anopheline mosquitoes were collected from random locations in three provinces of Pakistan during September-October 1989. A total of 95 collections were made by dipping larvae from breeding sites and aspirating adults from resting sites and animal bait traps. Blood-fed females were placed in labeled, individual 9-dram plastic vials with a screened top and provided water on which to lay their eggs. Those which survived hatching were individually reared in the laboratory. Field-collected larvae were reared individually in labeled, 9-dram plastic vials, also in the laboratory. Associated larval (4th instar) and pupal cast skins were preserved in 70% ethyl alcohol and the adults pinned. The adult and associated rearing skins were assigned a unique identification number. Taxonomic review of the specimens collected were determined by the Walter Reed Biosystematics Unit (WRBU) at the Smithsonian Institution, Washington, D.C.

On-site training in the conduct of enzyme-linked immunosorbent assays (ELISAs) for blood meal identification (host preference) and detection of malaria sporozoites [9, 10, 11] were completed. These tests were applied to adult anophelines collected from 6-8 urban and rural sites at locations north of Islamabad, south and central Rawalpindi. The collections were made routinely over a 12-month period using standard procedures for aspirating mosquito adults from human and animal shelters. All anopheline specimens were identified and processed for malaria sporozoite detection. In addition, blood engorged females were also processed for blood meal identification. The information from these preliminary surveys will be used for selecting sites for long-term studies and continued surveillance.

Results

About one-third of the specimens collected in 1989 have been identified by WRBU, and the results are shown in Table 1. Of interest are the collections of *An. willmori* which is an efficient vector and has not, to date, been recognized as a separate species and potential vector by malaria control workers in Pakistan. It is closely related to and easily confused with *An. maculatus,* and sophisticated taxonomy training is required to separate the two species [12].

Of 900 mosquitoes collected from different areas of Pakistan, 830 were analyzed by ELISA for blood meal and sporozoite determinations. All except two were found negative for oocyst and salivary stages of *Plasmodium vivax* and *P. falciparum*. These two, both *An. stephensi*, were taken from Nur-pur a small village just north of Islamabad. ELISA assay results for the mosquitoes collected from urban and rural locations are shown in Tables 2 and 3.

Discussion and Conclusions

Little can be stated at this point about the taxonomic survey until the entire collection has been identified. We anticipate this will be accomplished prior to the end of 1991. The finding of *An. willmori* is of considerable importance, and future efforts should be made to determine its overall abundance and distribution in Pakistan as well as its role in malaria transmission, if any. *An. willmori* is one of nine subspecies in the *An. maculatus* complex. The type locality is the Kashmir part of Pakistan, and the early specimens taken from there were positive for malaria [12].

Table 1. Mosquitoes identified from collections made in Pakistan during 1989.

I. Immatures (unmounted at present)

a. Vials of preserved whole larvae..... 13

b. Vials of preserved larval and pupal skins..... 135

2. Adults (pinned and labelled) as shown below:

Species	Reared with skins		Females	Total
	Male	Female	Biting	
1. *Aedes albopictus*			01	01
2. *Anopheles annularis*			05	05
3. *An. culicifacies*	05	10	25	40
4. *An. fluviatilis*	01	01	06	08
5. *An. lindesayi*	01			01
6. *An. maculatus*			02	02
7. *An. splendidus*	01	02	02	05
8. *An. stephensi*	12	16	11	39
9. *An. subpictus*		04		04
10. *An. willmori*	05	02	20	27
11. *Culex fuscocephala*	02	05		07
12. *Cx. quinquefasciatus*	12	17		29
13. *Cx. theileri*	04	02		06
14. *Cx. tritaeniorhynchus*	14	12		26
15. *Cx. vishnui* complex	03	04		07
16. *Cx. fuscanus*	01			01
Total Pinned Mosquitoes	61	75	72	208

Table 2. ELISA bloodmeal assay results for mosquitoes collected from urban and rural areas of Pakistan.

Species of Anopheline	No.	Human	Bovine	Goat	Sheep	Chicken
An. culicifacies	499	26	10	42	27	2
An. fluviatilis	94	09	02	11	03	0
An. splendidus	02	0	0	02	0	0
An. stephensi	160	04	02	09	14	0
An. sergenti	07	0	01	0	0	0
An. maculatus	29	05	03	10	07	0
An. annularis	12	0	0	02	01	0
An. subpictus	27	0	0	03	03	0
TOTAL	830					

Table 3. ELISA sporozoite assay results for anopheline mosquitoes collection from urban and rural areas of Pakistan.

Species of Anopheline	No.	Oocyst Stage	Salivary Stage
An. culicifacies	499	-ve	-ve
An. fluviatilis	94	-ve	-ve
An. splendidus	02	-ve	-ve
An. stephensi	160	-ve	2+ve
An. sergenti	07	-ve	-ve
An. maculatus	29	-ve	-ve
An. annularis	12	-ve	-ve
An. subpictus	27	-ve	-ve
TOTAL	830		

Literature Cited

1. Ghauri, A.W.K. and A.H. Munir. 1978. Problem of malaria control. Pak. AF Med. J. XXIX, No. 3:108-125.

2. Reisen, W.K. and M. Aslamkhan. 1978. Biting rhythms of some Pakistan mosquitoes (Diptera: Culicidae). Bull. Ent. Res. 68, 313-330.

3. Reisen, William K., Farida Mahmood and Tauheeda Parveen. 1979. *Anopheles subpictus* Grassi: Observations on survivorship and population size using mark-release-recapture and dissection methods. Res. Popul. Ecol. 21(1):12-29.

4. Reisen, William K., and Muhammad Aslamkhan. 1979. A release-recapture experiment with the malaria vector, *Anopheles stephensi* Liston, with observations on dispersal, survivorship, population size, gonotrophic rhythm and mating behavior. Annals of Trop. Med. Parasitol. 73(3):251-69.

5. Reisen, William K., Farida Mahmood and Tauheeda Parveen. 1980. *Anopheles culicifacies* Giles: a release-recapture experiment with cohorts of known age with implication for malaria epidemiology and genetical control in Pakistan. Trans. Roy. Soc. Trop. Med. and Hyg. 74(3):307-317.

6. Reisen, William K., Farida Mahmood and Khawar Azra. 1981. *Anopheles culicifacies* Giles: Adult ecological parameters measured in rural Punjab Province, Pakistan using capture-mark-release-recapture methods, with comparative observations on *An. stephensi* Liston and *An. subpictus* Grassi. Res. Popul. Ecol. 23 (1), June 1981:39-60.

7. Reisen, William K., Richard K. Sakai, Richard H. Baker, Khawar Azra and Shsheen Niaz. 1982. *Anopheles culicifacies*: Observations on population ecology and reproductive behavior. Mosq. News 42(1):93-101.

8. Ahmad, N., Z.K. Tariq. 1967. High altitude malaria prevalence in Gilgit Agency. Parkistan J. Science 19(5 & 6):199-204.

9. Burkot, T.R., F. Zavala, R.W. Gwadz, W.E. Collins, R.S. Nusenzweig and D.R. Roberts. 1984. Identification of malaria sporozoite-infected mosquitoes by a two-site enzyme-linked immunosorbent assay. Am. J. Trop. Med. Hyg. 33(2):227-31.

10. Wirtz, R.A., T.R. Burkot, R.G. Andre, R.M. Rosenberg, W.E. Collins and D.R. Roberts. 1985. Identification of *Plasmodium vivax* sporozoites in mosquitoes using an enzyme-linked immunosorbent assay (ELISA). Am. J. Trop. Med. Hyg. 34:1048-1054.

11. Burkot, T.R. and R.A. Wirtz. 1986. Immunoassays to detect and identify sporozoites in mosquitoes. Rev. Parasitol. Today. 2:155-57.

12. Harrison, Bruce. 1990. Personal communication.